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# *The Journal of Laboratory and Clinical Medicine*

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## *CLINICAL AND EXPERIMENTAL*

### AN EXPERIMENTAL STUDY OF THE LYMPHOCYTIC RESPONSE IN THYROTOXICOSIS\*

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POSITIVE evidence for the existence of a thyroid-lymphoid relationship is indicated by numerous clinical and pathologic observations (Plummer,<sup>1</sup> Falta,<sup>2</sup> and others) and by numerous experimental studies (Hoskins<sup>3</sup>; Kahn<sup>4</sup>; Gudernatsch<sup>5</sup>; Marine, Manley, and Baumann<sup>6</sup>; and Adams and Shevket<sup>7</sup>; and others), but positive evidence showing whether the lymphatic hyperplasia and consequent lymphocytosis is or is not functionally related to the hyperthyrosis is lacking; although, as Rowntree<sup>8</sup> points out, thymic hyperplasia in exophthalmic goiter has been considered by some as a compensatory hypertrophy, and Gudernatsch concluded that the thymus, spleen, and perhaps other lymphoid tissues as well, constitute glands with a positive internal secretion.

The present study was initiated by the constant observation by one of us (Turley) through thirty years of pathologic investigation that (1) hyperthyrosis is accompanied by a lymphocytosis; (2) the phenomenon of lymphocytic infiltration of the thyroid gland in thyrotoxicosis is interpretable in the light of the law of phagocytic infiltration, viz., that phagocytes infiltrate tissues at the site of pathologic processes; (3) all experimental work so far reported could be interpreted on a functional basis; and (4) the relation, whatever its nature, involved the lymphocytes as cells more than it did lymphatic tissues as organs. In view of these indications it seemed desirable that an attempt be made to determine whether the generalized lymphoid hyperplasia and the lymphocytes in thyrotoxic lymphocytoses are performing a function related to the thyrotoxicosis.

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## METHODS

The experimental animals used were guinea pigs weighing between 300 and 500 Gm. each. The animals were divided into three groups. In group I each animal was fed a balanced diet to which was added either 1 or 4 grains of thyroid substance daily. In group II each animal was fed daily a normal diet plus 1.5 Gm. of alenronat. In group III each animal received daily 1 grain of thyroid and 1.5 Gm. of aleuronat plus a normal diet. Four animals were used in group I, 6 in group II, and 8 in group III.

Total and differential blood counts, hemoglobin estimates, and weighings were made daily on each animal. As nearly as possible, blood was taken from each animal at the same hour every day. To avoid a generalized traumatic reaction in the ear and its possible effects on the blood picture, a single ear vein was severed in the right and left ears on alternate days. When deaths occurred, autopsies were done and tissues of all pertinent organs were taken for microscopic study.

In a preliminary series of studies the animals of groups II and III contracted pneumonia during the course of experimentation. This complicated our results and made it difficult, if not impossible, to distinguish between the immediate effects of the drugs administered and the effects of the pneumonic condition. Because of this complication, and because of the positive indications of these experiments, a second series of groups II and III experiments were performed, during which our animals were maintained free of intercurrent infection. In the following discussion of these studies, all remarks concerning groups II and III have reference to these second series unless definitely stated to pertain to the first and preliminary groups II and III experiments.

## RESULTS

Group I: The average survival time of these animals was three days. Of this group animals 1 and 2, receiving 1 grain of thyroid daily, lived 3.2 days; and animals 3 and 4, receiving 4 grains daily, lived only two days (Fig. 1A).

All the animals lost weight rapidly. Those receiving 1 grain of thyroid lost 29.5 Gm. daily, and those receiving 4 grains lost 72.5 Gm. daily (Figs. 2, 3A).

Erythrocyte counts varied individually and according to the amount of thyroid substance administered. The number of red blood cells increased in those animals receiving 1 grain of thyroid substance and decreased in those receiving 4 grains (Fig. 2).

Four prominent alterations in the leucocytic formula occurred. (1) In all but one animal a persistent leucopenia developed. (2) In all animals lymphocytes were dominant, both absolutely and relatively, during experimentation. (3) All but one animal showed an absolute but variable increase in the number of lymphocytes at the end of the first experimental day, and on all subsequent days an irregular but absolute decrease in lymphocytes occurred. (4) In every case the number of neutrophils decreased during experimentation (Fig. 2). Significant alterations in the number of eosinophiles, basophiles, and monocytes did not occur.

Group II: No deaths occurred during the course of experimentation which lasted thirty-two days (Fig. 1). Animal 4 was sacrificed for tissue and autopsy on the fifty-first day, and the others on the 120th day. The autopsy material appeared negative.

Except for an occasional variation, all the animals in this group gained weight during the course of experimentation. Animal 2 developed a temporary infection and was disregarded in our interpretations. Animals 1, 3, and 4, considered collectively, gained weight at the rate of 1.3 Gm. daily (Figs. 3A and 5). Weight changes in these animals occurred in diphasic cycles; that is, for a period of one to three days weight increases of 3 to 10 Gm. daily occurred, and then for a period of about the same duration little or no weight would be gained, or a little might be lost. Such diphasic cycles are repeated (Figs. 5, 6).

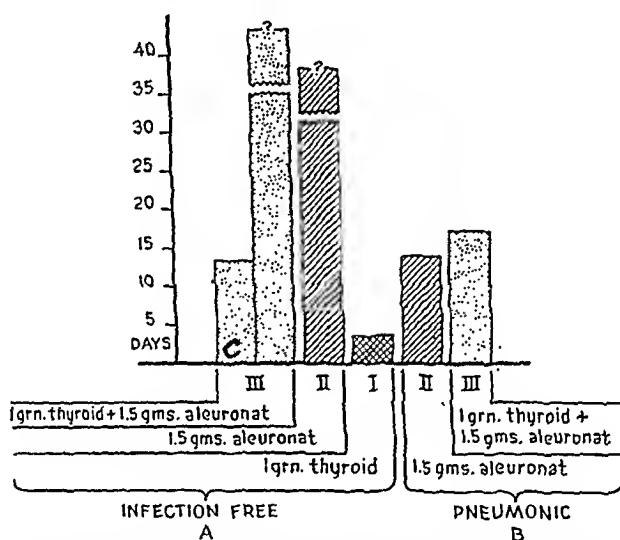


Fig. 1.—A comparative chart showing the survival time in days of the animals of experimental groups I, II, and III. A, second series of experiments which were free of intercurrent infection; B, preliminary experiments in which pneumonia intervened; C, animal 3, the only group III animal to die of this treatment.

Alterations in the peripheral blood were pronounced. The number of red blood cells varied characteristically from day to day and with a moderate increase (Figs. 5, 6). Although the total number of white blood cells showed daily wide variations, the average daily count for animals 1, 3, and 4 during thirty-one experimental days was only 6 per cent above normal (Figs. 5, 6). Changes in the leucocytic formula were the most pronounced and were characterized by an absolute and persistent increase in the number of lymphocytes and by an absolute decrease in neutrophils (Figs. 4, 5, 6). There were no changes in the number of other blood cell types from normal.

The developmental sequence of these alterations in the leucocytic formula can be easily discerned from the graphs, but it is desirable to point out, first, that there is an immediate rise in the number of lymphocytes, and that by the sixth experimental day the absolute number of lymphocytes may be from one and five-tenths to three times larger than the normal number (Figs. 5, 6); and second, that marked leucocytoses occur rhythmically in cycles which, with a fair degree

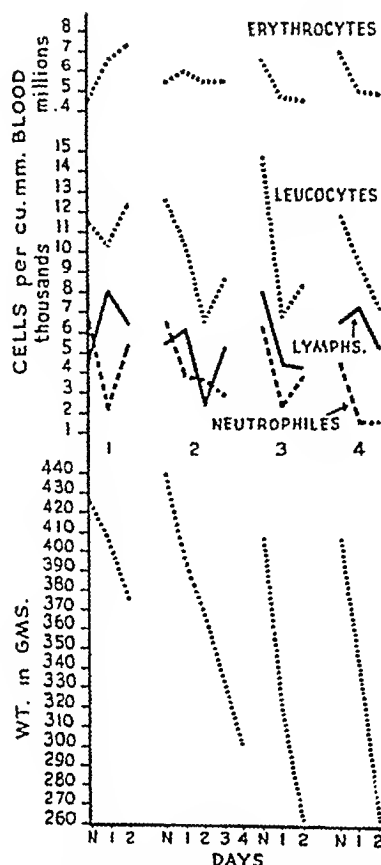


Fig. 2.—A graph of the daily alterations in weight and in the blood picture of the group I animals. Animals 1 and 2 were fed 1 grain of thyroid daily; animals 3 and 4 were fed 4 grains of thyroid daily.

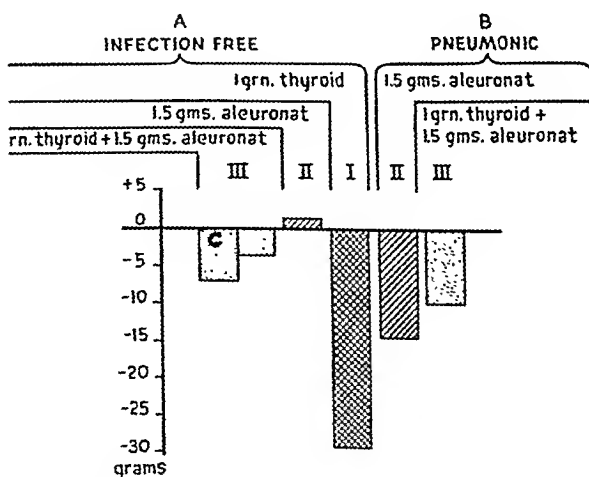


Fig. 3.—A comparative chart showing the average daily rate of weight change in animals of experimental groups I, II, and III. A, second series of experiments which were not complicated by intercurrent infection; B, preliminary experiments in which pneumonia intervened; C, animal 3, the only group III animal to die of this particular treatment.

of accuracy, coincide phase by phase with the cyclic weight changes mentioned above, and that there is an even closer correlation between cyclic lymphocytoses and the weight changes (Fig. 6).

Group III: All but one of the group III animals receiving lethal doses of thyroid substance in conjunction with aleuronat lived for an indefinitely long time. Only animal 3 died during the course of experimentation, but it lived thirteen days. The exact length of time that the other animals could have survived this treatment is not known. Animals 1 and 6 received these drugs for thirty-one days, and animals 2, 4, and 5, for thirty-nine days, without showing evidence of impending death, and in immediately subsequent experiments performed on them, lasting from twenty to sixty days longer, death did not result (Fig. 1).

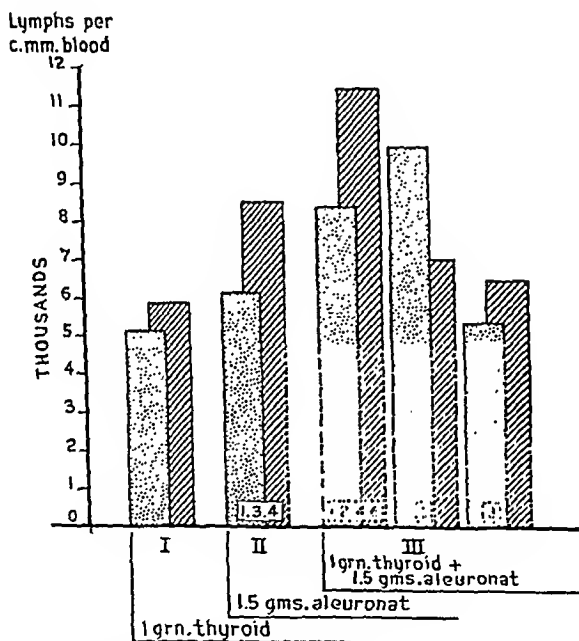


Fig. 4.—A comparative chart showing the average number of lymphocytes per cubic millimeter of blood per experimental day (diagonally lined columns) and the average normal number of lymphocytes per cubic millimeter of blood (stippled columns) of the animals of groups I, II, and III.

The animals in our preliminary group III experiments who contracted and actually died of pneumonia lived 16.5 days (Fig. 1B).

In this group the weight changes of every animal were basically alike, being characterized by three common alterations. (1) For a varying number of days each animal progressively lost weight, until about one-third of its original weight was lost. (2) This was followed by an indefinite number of days during which the body weight was stabilized and characterized by minor weight gains and subsequent losses to the basic low-weight level. (3) Weight loss occurred rhythmically in diphasic cycles occupying a varying number of days, usually three to seven. The first phase is characterized by either a slight loss or gain, or no change in weight, and the second by a marked weight loss exceeding the weight changes of the first phase whatever they may be (Figs. 7, 8). The col-

lective daily weight loss of animals 1, 2, 4, 5, and 6 was 3.5 Gm. Animal 3, which died in thirteen days, lost 6.9 Gm daily, which is double the weight lost by the others (Fig. 3A).

The average daily weight loss of our preliminary group III animals was 10 Gm. (Fig. 3B).

The number of red blood cells with daily variations increased above normal (Figs. 7, 8). There was much daily variation in the total number of white blood cells. The average white blood cell count of animals 1, 2, 4, and 6 during the course of experimentation was only 6 per cent lower than normal. Alterations in the leucocytic formulas of these animals showed the closest agreement possible. For the first few days (one to five) the number of lymphocytes remained near the normal level or slightly below it, but this period was followed by a marked rise in the lymphocytes so that, inclusive of both periods, the average number of lymphocytes per experimental day was 35.7 per cent above normal (Figs. 4, 7, 8). The neutrophils increased slightly during the first two days of experimentation, but they later decreased considerably below normal to a stabilized level. The average number of neutrophils per experimental day was 36.8 per cent below normal (Figs. 7, 8).

Animal 5, although unknown to us in the beginning, had a preexperimental existing pathology. Its total white blood cell count before experimentation was 12,960, of which 76.5 per cent were lymphocytes and 19.5 per cent were neutrophils. During the course of experimentation, the total white blood cell count was on the average 35.7 per cent below this animal's normal, but this was due mostly to a 29.3 per cent (in absolute terms 2,914 cells per cubic millimeter) decrease in the number of lymphocytes, and to a lesser extent by a 47.7 per cent (1,320 cells per cubic millimeter) decrease in the neutrophils. After suspension of the experimental feeding, the blood picture returned to the normal for this animal.

The total white cell count of animal 3 was reduced 12.2 per cent during the course of study. Alterations in the leucocytic formula were like those of animals 1, 2, 4, and 6 for the first six days, but on the seventh day the neutrophils increased to near the normal number, and for the remainder of the time that this animal lived, the leucocytic formula was practically normal.

In all animals of this group lymphocytoses occurred rhythmically in diphasic cycles which coincided phase by phase with concurrent cyclic weight changes (Fig. 8).

Our experimental method was built upon the proposition that if lymphocytes were functionally related to a hyperthyroid condition, an added concurrent stimulation of lymphopoieses and lymphocytosis in an experimental hyperthyroid state should mitigate the effects of this hyperthyroid state. Experimental bases of comparison were established by the experimental series of groups I and II.

Our group II series of experiments showed that aleuronat when taken by mouth induces a generalized lymphoid reaction in which a persistent and high lymphocytosis is characteristic of the peripheral blood. The exact modus operandi of aleuronat in effecting lymphocytogenesis is unknown, but it seems

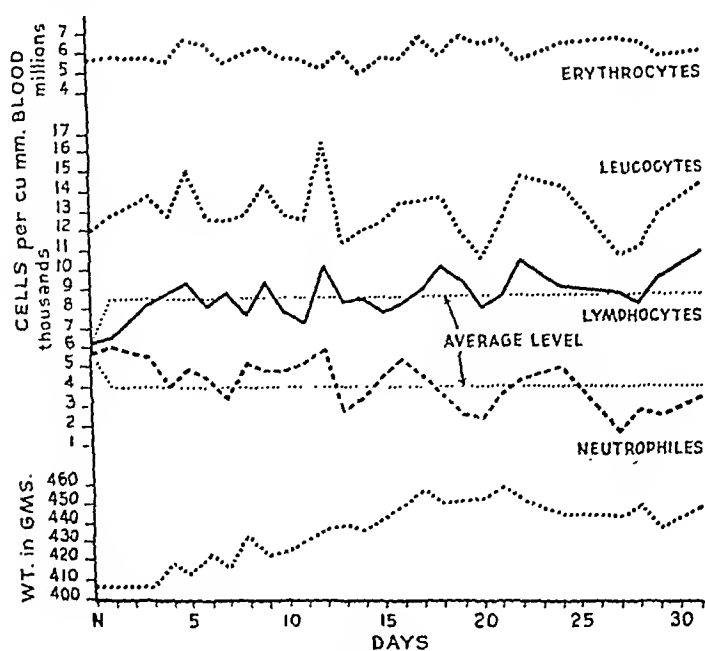


Fig. 5.—A composite graph of the daily alterations in weight and blood picture of animals 1, 3, and 4 of group II which were fed aleuronat.

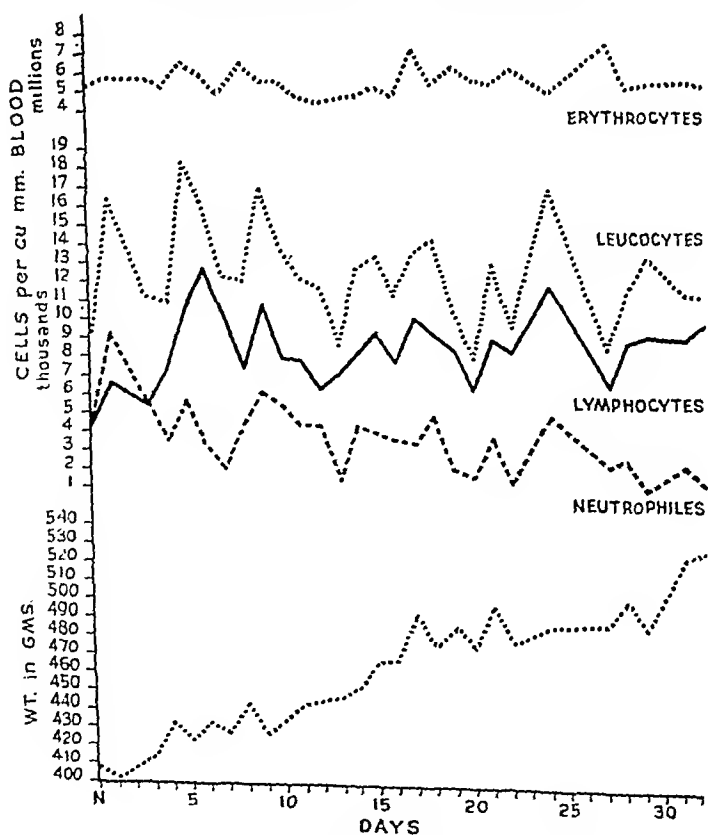


Fig. 6.—A graph showing the daily alterations in weight and blood picture of animal 3 of group II.



to be something of a toxic reaction. If weight loss may be used in this instance to indicate toxicity, it is evident that the aleuronat in the amounts given was only slightly toxic because the animals in this series actually gained weight during the greater part of these experiments which lasted thirty-two days, and when weight loss did occur, it was so slight and of such short duration that it seems negligible and to have little bearing on the final interpretation of our results.

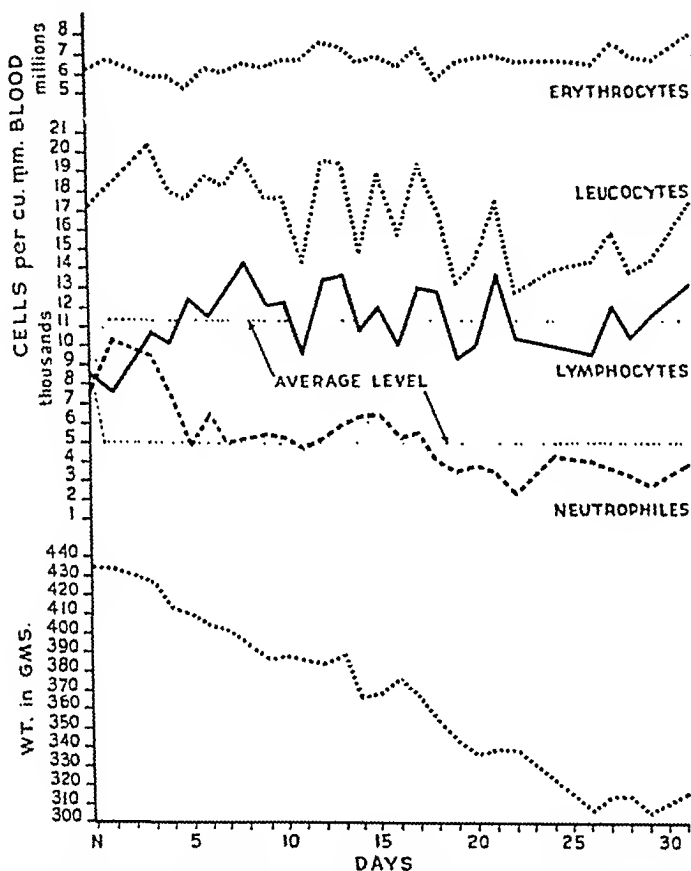


Fig. 7.—A composite graph showing the daily alterations in weight and blood picture of animals 1, 2, 4, and 6 of group III which were fed thyroid and aleuronat.

Our group I series of experiments showed that the hyperthyroid state induced by the daily administration of 1 grain of thyroid substance causes death after three days of treatment; that death is preceded by marked daily weight losses, and by a progressive decrease in the degree of lymphocytosis on days subsequent to the first experimental day which varies directly with the amount of thyroid given. The latter phenomenon is subject to many interpretations, but we believe that the lymphocytes are "used-up" in some manner faster than they can be produced. We rather doubt that the reduction in the number of lymphocytes in peripheral blood is due to a premature death of the lymphocytes because of an increased metabolic effect of thyroxine, because thyroxine seems unable to increase metabolic activity of lymphoid tissue (Anselmino, Eichler, and Schlossmann<sup>9</sup>).

With this knowledge of the individual effects produced by the daily administration of 1 grain of thyroid and 1.5 Gm. of aleuronat, an interpretation becomes possible of the results obtained in our group III series of experiments, in which both of these drugs in the amounts just indicated were administered jointly. The results of these group III experiments showed that, although 1 grain of thyroid substance daily did induce a marked hyperthyroid state in these

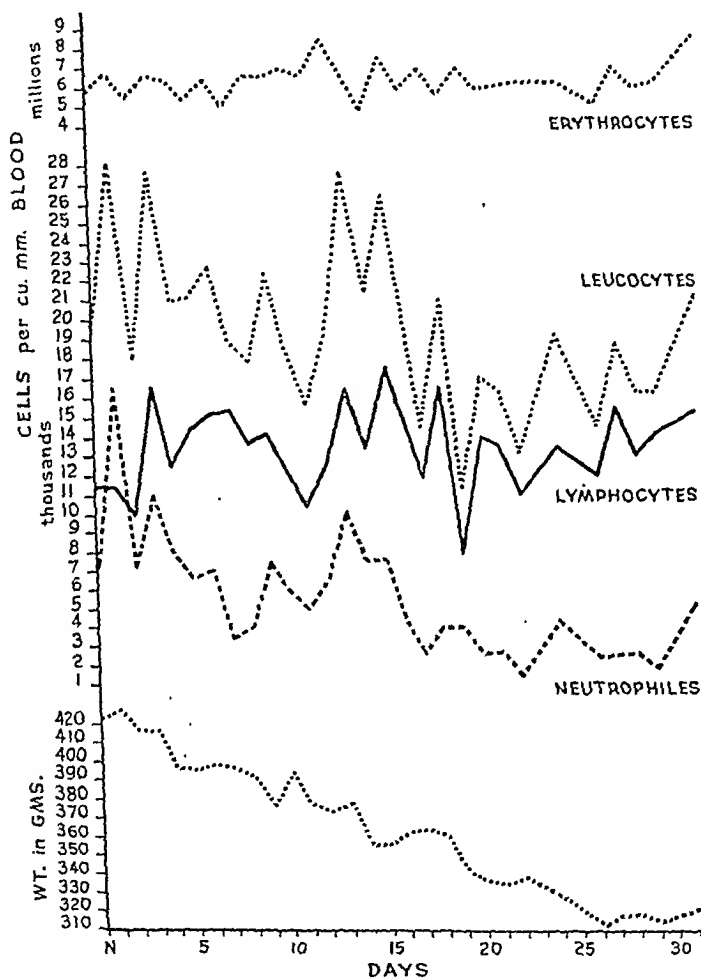


Fig. 8.—A graph showing the daily alterations in weight and blood picture of animal 1, group III.

animals when administered in conjunction with aleuronat, the severity of the hyperthyrosis was considerably less than that produced when this amount of thyroid substance was given alone, as in group I. In view of the facts that all the animals in group III lived longer, and, with the exception of one animal, lived indefinitely longer than did those of group I; and that the rate of weight loss experienced by these animals was but a fraction of that of the animals of group I; and that even the animals of our preliminary group III experiments who contracted and actually died of pneumonia lived five times longer and lost weight more slowly than the animals of Group I; the inevitable conclusion is,

that the mitigation of the known potential fatal effects of 1 grain of thyroid substance when taken daily must have been due to the lymphocyto-genic effect of the aleuronat, and that the lymphocytes constituted the means by which this mitigation was accomplished. It would seem that this inference is correct for several additional reasons. (1) In all group III animals there was an almost direct correlation phase by phase of cyclic lymphocytoses with cyclic weight losses which showed that when the number of lymphocytes increased, the rate of weight loss decreased; and when the number of lymphocytes decreased, the rate of weight loss increased. In group II such a correlation is also evident, for the rate of weight gain is greater when the number of lymphocytes increases; and conversely, when the number of lymphocytes decreases, the rate of weight gain decreases. (2) The only death occurring among the group III animals involved an animal whose hemopoietic system failed to respond as expected on the basis of the group II experiments to aleuronat stimulation. (3) Animal 5, group III, which before experimentation had an existent pathology requiring a high lymphocytosis was able to mitigate completely the fatal effects of lethal doses of thyroid substance even in the face of a persistent and lower degree of lymphocytosis which occurred during the course of experimentation. However, the total lymphocyte count of this animal, in spite of this reduction, was above that of the animals of group I and above that of animal 3 of group III which died because of a poor lymphocytic response. (4) In supplementary experiments on two animals in group III to test the effects of continued thyroid feeding alone, a lymphocytosis was maintained at a high level. (5) Death occurred in every instance when the time rate and degree of lymphopoietic response was less than the time rate and degree of development of the thyrotoxicosis. (6) It would seem further that the mitigation was due directly to the lymphocytes rather than to any reaction between aleuronat and thyroxine, or to the action of antihormonal substances, because two animals of group III which were fed thyroid in the absence of aleuronat for thirty days or longer in supplementary experiments showed no augmented thyrotoxic symptoms; and when the dose of thyroid was suddenly increased, death occurred as promptly as it did in those animals of group I.

#### CONCLUSIONS

(1) Aleuronat when taken by mouth, and in doses of 1.5 Gm. daily, induced a characteristic lymphocytosis in the blood of guinea pigs weighing between 300 and 500 grams each. This lymphocyto-genic effect of aleuronat may have been due to the fact that the drug was slightly toxic.

(2) The daily feeding of 1 grain of desiccated thyroid substance to half-grown guinea pigs produced a thyrotoxicosis which was fatal in from two to four days.

(3) The daily feeding of 1.5 Gm. of the lymphocyto-genic drug, aleuronat, in addition to a daily lethal dose (1 grain) of thyroid substance to half-grown guinea pigs either prevented completely or delayed the development of a fatal thyrotoxicosis. The evidence indicated that the living lymphocytes constituted the means by which this mitigation of the known potential fatal effect of daily 1 grain doses of thyroid was accomplished.

(4) The lymphocytes in thyrotoxic lymphocytosis are performing a function directly related to the alleviation of the thyrotoxicosis.

(5) It would further seem that the lymphocytic infiltration of toxic thyroid glands is an expression of this lymphocytic function.

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## A STATISTICAL STUDY OF TEMPERATURES IN HYPOGLYCEMIC COMA\*

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#### INTRODUCTION

THE use of hypoglycemic comas in the treatment of schizophrenic patients presents such striking clinical features that there is a temptation to generalize from observations during the treatments. This is particularly so if the observation is repeated in a number of different cases. One of the most significant physical changes induced by insulin hypoglycemia is low body temperature, the reading sometimes being below 90 degrees. The depth to which these temperatures will drop seems to vary greatly, but observations suggest that they bear some interesting constant features. It was noticed for instance that in some cases patients whose temperatures dropped very low during the early treatment periods did not reach such a low point during later treatments. It seemed, therefore, to be of some interest to determine whether a statistical analysis of a sufficient number of cases would yield any reliable data concerning this rise. We wished to determine whether it bore some definite relationship to the outcome of the treatment; whether it was related to the dosage of insulin used, or whether it was an individual expression of the patient treated.

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Patients were observed carefully during each shock period and individual manifestations of the shock were recorded on a chart previously described.<sup>7</sup> Dosage was regulated so that the patient was in coma at the end of three hours and had one hour of this for each treatment after the first ten treatments, which were usually less prolonged.

If the temperature drop during hypoglycemia is due to the size of the insulin dose, it may be that the compensatory rise depends on the adequacy of the autonomic centers to meet this strain. There is much to indicate that schizophrenic patients lack the ability to respond with such homeostatic mechanisms.<sup>3-5, 8, 9</sup> That hypoglycemia induces a compensatory sympathetic activity has been indicated by many authors.<sup>1, 6, 10</sup> We might expect, therefore, that the better patients would tend to be better able to combat the temperature drop; that as recovery took place the coma temperature would be closer to the normal temperature and the temperature would tend to keep constant regardless of the size of insulin dosage.

#### METHODS

In these studies the insulin injections were given at 6 A.M. and the patients were given glucose at about 10 o'clock or later. The temperature was taken at 10 o'clock before termination by means of glucose gavage. At this time most of the patients were in coma. Rectal temperatures were used throughout this study.

The daily 10 A.M. shock temperature records of 36 patients who recovered after insulin shock were studied. This group was considered "A" recoveries and will be referred to as group A throughout this study. Twenty patients who failed to show any improvement and are, therefore, designated as group D were likewise studied.

The first forty-five days were studied in each case since many patients were not treated longer than this. The first statistical study consisted of obtaining a mean of the 10 o'clock temperature, for all patients of each group, and for each day of the forty-five days.

The range of daily means for the coma temperatures of group A were from 96.79 for the first day of treatment to 97.02 for the forty-fifth day of treatment. Although the means did not show a steady course, there was a definite trend upward. In the D group, although the mean for the first day was 97.04 and for the last day 96.7, the daily means fluctuated so unsteadily that there was no constant trend.

When the temperatures were grouped for periods of one week or five days, the same trend was noticed. The averages for group A for the first and ninth weeks were 96.67 and 96.78, respectively. For the D group these means were 96.53 and 96.54. It must be remembered that each of these means represents five days' average for a large number of different patients. Although the trend upward in the A group may certainly be considered greater than that of the D group, there is statistically no reliable difference between the two.

A study of the units of insulin used for each treatment was then considered. Examining the amount of insulin used in the first dose (the shock dose), we find a mean of 104.03 for the A group, and a mean of 112.22 for the D group.

These differences are not reliable statistically. The shock doses of the two groups are approximately the same. Obtaining the means for the insulin given to the A group for each of forty-five treatments, we found the mean to be 104.03 on the first day of treatment and 76.1 on the forty-fifth day. In the D group these amounts were 112.22 to 83.33, respectively. There is a reliable difference between the dosage used at the beginning and end of treatment in group A, the insulin used being greatly diminished. In the D group the dosages were also diminished, although the difference is not as constant for all members of the group. On the whole, moreover, we note that the total insulin used on the A group for each of the forty-five days of treatment is less than that used on the D group.

TABLE I

## DAILY AVERAGES OF INSULIN DOSE AND TEMPERATURES IN HYPOGLYCEMIC SHOCK

| TIME OF TREATMENT           | TEMPERATURE |       | INSULIN |       |
|-----------------------------|-------------|-------|---------|-------|
|                             | MEAN        | SIGMA | MEAN    | SIGMA |
| <i>Group A, 36 Patients</i> |             |       |         |       |
| 1st day                     | 96.79       | 0.98  | 104.03  | 44.5  |
| 45th day                    | 97.02       | 1.17  | 76.11   | 42.9  |
| 1st week                    | 96.67       | 1.22  | 110.7   | 46.2  |
| 9th week                    | 96.78       | 1.33  | 75.2    | 41.7  |
| <i>Group D, 20 Patients</i> |             |       |         |       |
| 1st day                     | 97.04       | 1.10  | 112.22  | 61.4  |
| 45th day                    | 96.7        | 1.61  | 83.33   | 54.8  |
| 1st week                    | 96.53       | 1.38  | 118.7   | 68.7  |
| 9th week                    | 96.54       | 1.71  | 83.1    | 52.7  |

It might appear that the rise in temperature observed in improved patients in the course of the treatment may be due to the greater decrease in the amounts of insulin used in this group during the treatments as compared to that of group D. In order to see whether this is true, partial correlations between day of treatment, insulin dosage, and coma temperature, were made. These correlations were found to be so low that it can be said that no correlation exists between insulin dose and temperature.

TABLE II

## PARTIAL CORRELATIONS BETWEEN DAY, AMOUNT OF INSULIN, AND 10 O'CLOCK TEMPERATURE

| Group A, 1,364 Treatments |                   |                               |                                |
|---------------------------|-------------------|-------------------------------|--------------------------------|
|                           | (1) Day           | (2) 10 o'clock<br>Temperature | (3) Insulin                    |
| Mean                      | 23.15             | 96.6                          | 77.3                           |
| Sigma                     | 12.48             | 1.27                          | 38.6                           |
| Correlation               |                   |                               |                                |
| between (1) and (2)       | $= 0.13 \pm 0.02$ |                               | (2) and (3) $= 0.02 \pm 0.02$  |
|                           |                   |                               | (1) and (3) $= -0.27 \pm 0.02$ |
| Group D, 796 Treatments   |                   |                               |                                |
|                           | (4) Day           | (5) 10 o'clock<br>Temperature | (6) Insulin                    |
| Mean                      | 22.67             | 96.46                         | 94.7                           |
| Sigma                     | 12.75             | 1.61                          | 60.8                           |
| Correlation               |                   |                               |                                |
| between (4) and (5)       | $= 0.02 \pm 0.02$ |                               | (5) and (6) $= 0.06 \pm 0.02$  |
|                           |                   |                               | (4) and (6) $= 0.20 \pm 0.02$  |

A study of the temperature and insulin dose, considering all days for all patients of the A group compared to those of the D group, reveals some striking findings. The temperatures of those in group A were higher than those in group D. The reliability of this difference is 2.09, indicating a significant difference, which probably would be proved to be statistically reliable if more cases were considered. The amount of insulin used all forty-five days for patients of group A was less than that used in patients of group D, and this difference is statistically reliable.

#### DISCUSSION

We noticed that patients who recovered from schizophrenia after undergoing insulin shock therapy (group A patients) required less insulin during the course of treatment. This may suggest that patients requiring lower doses to induce shock may have a better chance of recovering than those requiring larger doses. On the whole, moreover, the group A patients do not reach as low temperature levels as do the patients who do not improve. Of interest is the demonstration in these studies that the group A patients show an increasing temperature during the course of treatment, with a tendency to return during insulin coma to normal temperature levels. The possibility suggests itself that this indicates an improvement in the homeostatic functions of these patients. That such improvement occurs has been suggested by other authors as demonstrated by improved blood pressure and other signs of cardiac functions.<sup>2</sup> It would be interesting if the probability of a good remission could be predicted during treatment by upward rise in the coma temperature curve. At any rate, the findings presented here indicate that the determination of temperature during insulin shock may be of definite importance beyond mere interest, and that the supposition that the temperature level observed during hypoglycemia is merely dependent on dosage of insulin is unfounded.

#### SUMMARY

1. Statistical studies of rectal temperatures taken during insulin coma are reported.
2. The range of daily means for 36 recovered patients showed a trend upward. Twenty unimproved patients showed no such rise.
3. Initial insulin shock doses were the same in both groups. There was a definite decrease in the daily insulin dosage used in the course of treatment, this decrease, however, being more constant in the A group.
4. We note, moreover, that the total insulin used on the A group is less than that used on the D group.
5. The rise of temperature in the course of treatment in the A group is not due to the decrease in the insulin dosage.
6. The temperature of the group A on the whole was greater than that of the group D.
7. The amount of insulin used for the group A patients for all the treatments was less than that used on the group D patients.

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## SEROLOGIC DIAGNOSIS IN HISTOLOGICALLY PROVED CHRONIC SYPHILIS\*

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### AN ANALYSIS OF 424 NECROPSIES

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ONE frequently finds contradictory figures given as to the percentage of positive serologic tests in cases of various types of chronic syphilis. It was for this reason that it was decided to analyze the autopsy protocols of the Detroit Receiving Hospital for the period from January, 1928, to July, 1940, in order to determine the incidence of positive serologic reactions in the various forms of proved autopsy syphilis.

The serologic tests performed were as follows: previous to November, 1933, Kolmer and Kahn tests; from November 1, 1933, to January 1, 1934, Kolmer, Kahn, and Kline tests; and from January 1, 1934, to July 1, 1940, the Kline test was used as an exclusion test, and Kahn tests were performed on those sera which gave positive Kline reactions.

No attempt was made to determine the relative merit of the serologic tests employed. If any one test was positive, it was considered sufficient reason for recording the serology as positive for syphilis. Results were reported as positive, negative, or doubtful.

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\*From the Pathological Laboratories of Receiving Hospital, Detroit.  
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In this study of autopsy protocols for the period mentioned, 555 persons with microscopically proved syphilis were encountered. However, of this group, only 424 patients had an accompanying serologic report. The remainder died in the admitting room or on the wards shortly after admission, and no opportunity was afforded of obtaining a specimen of blood.

TABLE I  
SEROLOGIC REPORTS

|          | CASES | PER CENT |
|----------|-------|----------|
| Positive | 321   | 75.7     |
| Negative | 82    | 19.3     |
| Doubtful | 21    | 5.0      |
| Total    | 424   | 100.0    |

Table I shows the serologic findings in the 424 patients included in the study. It is noted that 75.7 per cent of these gave positive serologic reports.

In attempting to compare the figures obtained at the Receiving Hospital with those frequently quoted, one finds that very often there is no common ground upon which this comparison can be made. Most of the figures cited in the literature are based upon studies of groups of living patients, whereas, the cases covered in this report were all proved by microscopic examination of autopsy material. Kolmer<sup>1</sup> contends that in 80 to 100 per cent of chronic syphilis the serology is positive, and Stokes<sup>2</sup> states that he found 67 per cent of 252 persons to have positive serology. However, these figures were not compiled from autopsied material.

In this series it is seen that in 82 of 424 persons the serology was negative; a further analysis of this group brought out the interesting fact that in 60 per cent of the 82 cases, only syphilitic aortitis was found at autopsy. This leads to the question: Does the presence of a positive serologic reaction depend upon at least a certain minimal involvement of the organs and structures, or do the accepted pathologic criteria of what constitutes syphilitic aortitis need revision? If the answer to the first part of the question is in the affirmative, it would seem when there is syphilitic involvement of several organs the percentage of positive laboratory tests for syphilis should be higher. Table II shows the results of such an analysis.

TABLE II  
RELATION BETWEEN EXTENT OF INVOLVEMENT AND PERCENTAGE OF POSITIVE TESTS

|  | CASES | PER CENT |
|--|-------|----------|
| Only one organ involved (usually syphilitic aortitis): |       |          |
| Positive   | 206   | 71.0     |
| Negative   | 66    | 23.0     |
| Doubtful   | 19    | 6.0      |
| More than one organ involved:                          |       |          |
| Positive   | 115   | 86.0     |
| Negative   | 16    | 12.4     |
| Doubtful   | 2     | 1.6      |

It can be seen that the incidence of positive tests in the group of patients with multiple involvement was 21 per cent higher than in those with a single organ involved, and that there was 14 per cent more in the former group

who gave a positive reaction than in the total group of 424 persons. This finding is far from conclusive, but, nevertheless, gives some basis for the contention that positive serologic evidence of syphilis is somewhat dependent upon the extent of demonstrable pathologic involvement.

#### CARDIOVASCULAR SYPHILIS

Cardiovascular syphilis formed a major portion of the material studied. Some evidence of cardiovascular involvement was found in a total of 335 patients, or 79.0 per cent. This figure is about 33 per cent higher than that given by Symmers,<sup>3</sup> who found evidence of vascular system syphilis in only 56 per cent of the 314 cases he studied. In this series syphilitic aortitis was the most common lesion encountered, occurring in 303 patients, or 71.4 per cent of the total (424 patients), a figure which is closely comparable to the one given by Cecil,<sup>4</sup> who maintains that syphilitic aortitis is present in 70 per cent of persons with chronic syphilis.

In analyzing the serologic reports of the cardiovascular syphilis group, it was found that 71.6 per cent gave positive serology. This figure is considerably below the one given by White,<sup>5</sup> who states, that "the serum reaction for syphilis is generally positive, and strongly so in cardiovascular syphilis; sometimes in approximately 15 per cent of the cases it is negative." Kolmer<sup>1</sup> claims that in chronic cardiovascular syphilis 80 to 96 per cent of patients will give positive serology by his method. Moore<sup>6</sup> found 85 to 95 per cent of his clinical cases to be positive. Christian<sup>7</sup> found that about 80 per cent of patients with syphilitic disease of the aorta gave a positive Wassermann reaction.

#### SYPHILIS OF ALIMENTARY TRACT

Thirty-five patients (8.2 per cent) had a diagnosis of some form of syphilitic involvement of the digestive tract. Table III shows the distribution of such lesions.

TABLE III  
SYPHILIS OF ALIMENTARY TRACT (35 CASES)

|           | POSITIVE   | NEGATIVE  | DOUBTFUL |
|-----------|------------|-----------|----------|
| Liver     | 21 (80.7%) | 4 (15.3%) | 1 (4.0%) |
| Pancreas  | 7 (77.7%)  | 2 (22.3%) | 0        |
| Esophagus | 1          | 0         | 0        |
| Midgut    | 1          | 0         | 0        |
| Cecum     | 1          | 0         | 0        |

Syphilis of the liver constituted only 6.1 per cent of the 424 patients in this series. Symmers<sup>3</sup> found the liver to be involved in 33 per cent of cases showing post-mortem evidence of syphilis. Brockbank<sup>8</sup> found the above to be true in 58 per cent of his cases. Flexner<sup>9</sup> found 88 cases of hepatic syphilis in 5,089 autopsies (1 per cent). McCrae<sup>10</sup> demonstrated 46 cases in 3,300 autopsies (1.3 per cent). The reason for the marked disparity between the figures quoted and those obtained in this series is not apparent.

Since the number of cases of syphilitic involvement of the liver<sup>26</sup> is comparatively small, the percentage figures obtained are indicative rather than conclusive. A total of 80.7 per cent had positive serology. McCrae and Caven<sup>11</sup> found

that 80.4 per cent of 41 persons with syphilis of the liver, which had been successfully diagnosed and treated, gave positive Wassermann reactions originally. O'Leary, Greene, and Rowntree<sup>12</sup> found that 90 per cent of their patients with hepatic syphilis had positive Wassermann reactions. Their diagnoses were made for the most part on the basis of clinical impressions and the effect of therapeutic tests.

#### CENTRAL NERVOUS SYSTEM SYPHILIS

Numerically, the group with syphilis of the central nervous system was second only to that showing cardiovascular syphilis. One hundred and thirty patients, or 30.6 per cent of the total, were found to have some form of syphilitic involvement of the central nervous system. No doubt this figure is much lower than the actual incidence of involvement of the central nervous system, since permission for examination of the cranial cavity of the patient was frequently not obtained. Of this number, 85.4 per cent had positive serology. Included in this group were 57 patients with general paresis, 87.7 per cent of whom had positive serology.

The figures given in the preceding paragraph differ from those given in reports by some well-known authors. Cecil<sup>4</sup> states that it is unusual and rare for the serology to be negative in syphilis of the central nervous system. Grinker<sup>13</sup> found the blood Wassermann positive in 65 per cent of all patients with neurosyphilis and that it may fall as low as 50 per cent in the latent phases of the disease. Kolmer's<sup>1</sup> figures ranged between 70 per cent and 100 per cent in his groups of patients with syphilis of the central nervous system. In general paresis, Grinker states, the blood serology is 100 per cent positive. Moore<sup>6</sup> found it 90 to 95 per cent positive. These figures are definitely higher than those found in the group of cases covered in this analysis.

#### SUMMARY

1. A total of 424 patients with microscopically proved syphilis encountered at autopsy were analyzed statistically. All of these had accompanying serologic examinations.

2. Of the entire group 75.7 per cent had positive serology.

3. The patients with multiple organs involved gave 21 per cent more positive reactions than those with only one organ involved.

4. A total of 335 patients (79.0 per cent) showed some evidence of cardiovascular involvement. Of these 71.6 per cent had positive serology.

5. There were 26 cases of hepatic syphilis. This amounted to only 6.1 per cent of all the cases. In 80.7 per cent of these the serology was positive.

6. One hundred thirty persons exhibited central nervous system involvement, and 85.4 per cent had positive serology. In 87.7 per cent of 57 persons with paresis the serology was positive.

#### CONCLUSIONS

1. The percentage of positive blood tests for syphilis for the entire group studied did not differ appreciably from that usually reported.

2. The percentage of positive reactions in cardiovascular syphilis was about 10 per cent lower than the figures that are usually given.

3. Demonstrable hepatic syphilis occurred 25 to 50 per cent less frequently than in many other series.

4. The percentage of positive tests in hepatic syphilis coincided closely with the experience of others.

5. The percentage of positive tests in general paresis was definitely below that found in other series.

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## THE DIAGNOSIS OF PERIPHERAL ARTERIAL OBSTRUCTION\*

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TRADITIONAL methods of examination for the diagnosis of peripheral arterial obstruction are becoming inadequate and uncertain because of the demand for more reliable and accurate information in this field of medicine. Sole reliance upon palpation of the dorsalis pedis pulse as the standard procedure in the diagnosis of circulatory disturbances in the lower extremities is rapidly losing its former worth.

Manual palpation of peripheral pulses may be misleading because of the following variable factors involved: (1) The skill and experience of the examiner. (2) Anatomical deviation in position of the peripheral arteries. (3) Edema or excessive adiposity of the extremities.

These objections apply not only to the dorsalis pedis pulse but also to the posterior tibial and popliteal as well. The familiar sight of a group of physicians in a vascular clinic disputing the presence or absence of a dorsalis pedis pulse is sufficient argument for the claim that individual skill and experience play a considerable part in this diagnostic procedure.

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Occasional instances of congenital absence of the dorsalis pedis artery have been observed. In these cases there is usually compensatory enlargement of a branch of the peroneal artery which produces pulsation in the lateral portion of the foot.

In the presence of edema of the feet either from cardiorenal or from infectious cause, the dorsalis pedis and posterior tibial pulses may be completely impalpable, although the arteries are patent. In obese patients similar difficulties may be experienced, not only with the arteries of the feet but with the popliteal as well.

Palpation of the popliteal artery is attended with considerable difficulty, and often requires various aerobic manipulations in order to feel pulsation of the vessel adequately. The status of the digital and plantar circulation of the lower extremities is not accurately revealed by palpation of the dorsalis pedis or posterior tibial arteries alone. If both of these vessels are felt in an extremity there is no assurance that the arterial tree distally is also patent. To ascertain this fact other tests must be utilized.

The importance of the detection of peripheral arterial disease in its early stages is obvious. Any method of examination which will reveal the presence of organic changes in the most distal parts of the extremity should be utilized more frequently than has heretofore been the rule. At the same time the physician who has relied traditionally solely upon palpation of the dorsalis pedis artery should realize the limitations of this method.

It is well to re-emphasize the fact that an important diagnostic point in the organic peripheral arterial diseases is the ability to detect the organic changes. The presence or absence of superimposed vasomotor disturbances, particularly vasospasm, is of secondary importance in diagnosis, the primary consideration being the detection of structural arterial disease. The tragedy of delayed diagnosis was recently illustrated in a case of thromboangiitis obliterans in a physician. About eight years ago he experienced symptoms in his legs which suggested to him the possibility of thromboangiitis obliterans. A physician was consulted who, after feeling both dorsalis pedis pulses and using no other diagnostic procedure, concluded that there was no evidence of arterial disease and that smoking might be resumed. A few years later the physician submitted himself for a blood count, for the determination of which it was necessary to puncture the tip of the right index finger. The trauma thus induced resulted in a gangrenous ulcer which did not heal until a year and a half later. At the first sign of gangrene the patient presented himself for an opinion. The lower extremities were examined and revealed definite right plantar ischemia in addition to poor dorsalis pedis pulses and excellent posterior tibial pulses. Oscillometric readings were 3.0 at the left ankle and 2.0 at the right ankle. Although both radial and ulnar pulsations could be felt, there was moderate ischemia of the fingers of both hands on elevation and exercise. There was a gangrenous ulcer at the tip of the right middle finger about 1 cm. in diameter. The patient refused to stop smoking, and about a year later no pulses could be felt in either foot and the oscillometric reading at the left ankle had decreased to zero. Extensive gangrene of the right index finger soon developed as well as gangrenous lesions of the left big toe and heel.

It is clear that had the diagnosis been made at the first examination, and had the patient been sufficiently impressed with the importance of complete cessation of smoking, the present situation could have been avoided. The assurance of the original examiner was so great that there was no arterial disease because of the bounding dorsalis pedis pulses that the patient was offered a package of cigarettes and told to smoke as much as he pleased.

In 1929 I<sup>1</sup> proposed a new test for the determination of arterial occlusion in the distal vessels of the extremities. This procedure, based on earlier observations of Oehler and Parkes-Weber, has proved so reliable over a period of fifteen years that it must be considered of diagnostic importance and as an obligatory part of the examination before the diagnosis of arterial disease is ruled out. Kramer,<sup>2</sup> from his large experience at the Philadelphia General Hospital, says "a positive plantar ischemia is significant. Our experience with this simple procedure has been that when positive findings are obtained, the other function tests will usually indicate evidence of an impaired peripheral circulation." Again, "we have found that a positive plantar ischemia has nearly always been associated with occlusive vascular disease." Lewin<sup>3</sup> also considers the test an important part of the routine examination for determining the circulatory efficiency of the lower extremities.

In carrying out the test the patient lies recumbent on the examining table, and the legs are elevated to an angle of about 60 degrees, supported on a stool or some other arrangement. While in this position the ankles are flexed and extended repeatedly for about a minute. During and immediately after the maneuver the soles of the feet are carefully examined in a good even light. In every instance of organic arterial disease, whether due to thromboangiitis obliterans, or arteriosclerosis obliterans, a characteristic pallor of the sole of the affected extremity will become apparent either during the motion of the feet or immediately afterwards. The pallor, or plantar ischemia, is usually in direct proportion to the degree of obstruction present. In incipient cases, particularly those with patent dorsalis pedis and posterior tibial arteries, the ischemia may be difficult to detect by the novice, but experience will soon help in judging relative differences in color.

In the more advanced cases no difficulty should be experienced in detecting plantar ischemia. A cadaverous pallor, which may involve the entire foot as high as the ankle or farther, becomes apparent almost immediately.

The same principle of inducing ischemia as a diagnostic method may be applied to the upper extremities. Elevation of the hand above the head with alternate opening and closing of the fingers will produce, after a few moments of this motion, a transient ischemia of the digits or hand deficient in blood supply. This test may be positive in incipient cases of organic arterial disease in which the radial and ulnar pulses can be felt. It is of more practical value than the modification proposed by Allen<sup>4</sup> which merely serves to distinguish between closure of the radial or ulnar artery. The test here described is more suitable for detecting early involvement of the digital arteries of the upper extremities.

Temperature changes are usually very pronounced in organic arterial disease even in the earliest stages. As a rule, the ischemic extremity is cool.

respondingly colder than the uninvolved limb. Where the condition is bilateral both will be colder than usual, with proportionate differences in coldness dependent upon the degree of occlusion present. For ordinary clinical diagnosis instrumental measurements are not necessary for temperature determination, the palm or back of the hand being sufficiently sensitive. For precise measurements of the skin temperature, the Dermalor<sup>5</sup> is preferred.

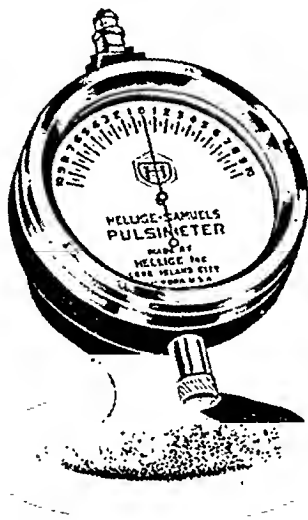


Fig. 1.—Hellige-Samuels pulsimeter.

#### OSCILLOMETRY

The observation of Cawadias in 1912 that the Pachon oscillogram was admirably suited to the study of arterial patency in the extremities opened up a new and interesting field. Visual observation of arterial pulsation was made available so that quantitative measurement of the pulse amplitude in the extremity could be easily determined. Furthermore, pulsation could be detected at points where by manual palpation the arteries were not accessible. The examining cuff could be placed at any point on the limb, and oscillations were recorded at that particular level, whereas by the manual method one is limited to only a few areas.

While the clinical diagnosis of arterial obstruction can be made without instruments, it is also true that a more accurate picture is conveyed by oscillographic determinations than by purely clinical methods alone. Other valuable information can also be obtained from the oscillogram. If one remembers that the pulsation obtained with the instrument represents the expansion of the main vessels only, deductions can be made therefrom concerning the state of the collateral circulation in the extremities in an indirect way. For instance, a negative reading at the ankle level indicates complete obstruction of the main arterial channel. This being the case, it follows, therefore, that the collateral circulation in the extremity must be very small. On the other hand, a positive reading at the same level indicates only partial occlusion of the main vessels and a correspondingly better collateral circulation. For prognostic study such observations are extremely important because a zero reading at the ankle level in a case

of arterial disease would naturally convey the impression that the ultimate recovery of gangrene, for instance, would be doubtful because of the poor collateral circulation. On the other hand, a positive reading gives more encouragement to conservative therapy with better chances for eventual healing.

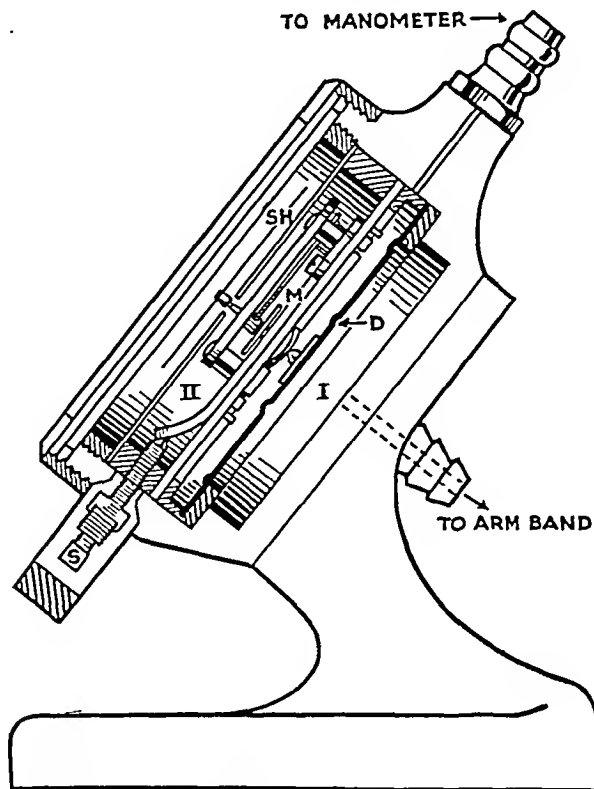


Fig. 2.—Schematic drawing to show construction of pulsimeter. *D*, aneroid diaphragm; *M*, mechanism for transmitting pulsation to indicating hand *SH*; *S*, set screw for adjusting indicating hand to zero; *I*, *II*, air chambers separated by diaphragm *D*.

Likewise, in cases without ulceration and gangrene zero readings mean more difficulty in conservative treatment, a longer period of therapy to attain symptomatic relief, and a doubtful outcome as far as ultimate development of gangrene is concerned. Positive readings in these cases mean better therapeutic results and less chance for the development of gangrene.

The oseillometer has thus extended the possibilities in the field of peripheral vascular diseases and should be made more available so that more accurate studies can be made and more practical information can be obtained. One of the great hindrances to the more universal use of the instrument has been its comparatively high cost. To overcome this objection a simplified apparatus has been devised which does not entail a large financial investment. This apparatus, because of its primary purpose of measuring pulsation, is redesignated "pulsimeter."

By means of a short piece of rubber pressure tubing, either an aneroid or a mercury sphygmomanometer is attached to the upper nipple of the pulsimeter. One outlet of the arm band of the blood pressure apparatus is connected to the



rear nipple of the pulsometer. It is essential that all connections be absolutely tight, so that there will be no leakage of air. If the inflating bulb of the blood pressure apparatus or the rubber arm band or any parts of the connecting tubes are defective, or allow even the slightest loss of air pressure, the pulsometer will not operate properly.

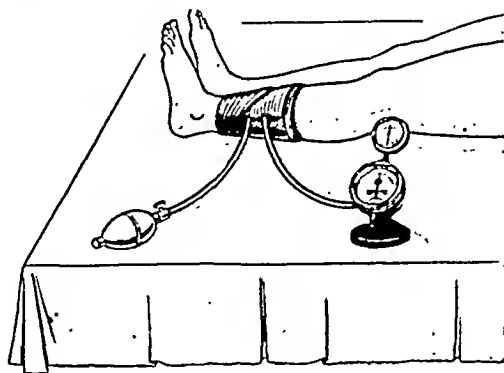


Fig. 3—Method of applying pulsometer for ankle readings.

In carrying out an examination, it is essential that the patient lie flat, completely relaxed, with the head back and arms at the side. If the patient is not completely relaxed, the slightest muscular movement, such as lifting a hand, will cause deflections of the pulsometer needle, interfering with the proper operation of the apparatus. The arm band of the sphygmomanometer is carefully applied, as in taking blood pressure, to the part under examination. As a rule, the region just above the ankle gives the most information if the lower extremities are being studied. It is essential that the cuff be applied snugly, not too tightly and not too loosely. Caution must be taken that there are no kinks in the tubing after the cuff is applied.

After all connections are tight and the cuff is properly applied, the escape valve on the inflating bulb of the blood pressure apparatus is closed tightly and the entire system is inflated. By watching the sphygmomanometer, the pressure being applied can be measured. During inflation, the indicating hand of the pulsometer will rotate a few times. This does not interfere with the operation of the apparatus and should be disregarded until the final inflating point is reached. The pressure in the cuff is increased until it is reasonably sure that the patient's systolic pressure has been exceeded. For routine examinations, it is sufficient to inflate the cuff to about 200 mm. of mercury, as indicated on the sphygmomanometer dial. When this point is reached, the indicating hand of the pulsometer revolves slowly until the entire system becomes stabilized. It is necessary to wait a few moments for this to happen before readings are taken.

As a rule, a pressure of 200 mm. of mercury will completely obliterate all pulsations; therefore, the indicating hand will not oscillate. In order to estimate the maximum oscillation of the indicating hand, it is necessary to decrease the pressure in the whole system by about 10 mm. at a time. This is done by partially releasing the air escape valve on the inflating bulb. With each deflation, the indicating hand may again revolve once or twice. It is important

to allow the hand to stabilize after each reduction in pressure in order to obtain a reading. As the pressure in the apparatus is decreased in stages of 10 mm. of mercury, the oscillations of the indicating hand increase in amplitude, until a point is reached corresponding to the patient's systolic pressure. When this level is obtained, the maximum oscillation known as the oscillometric index can be read.

It is not necessary that the indicating hand remain at the zero point either before, during, or after an examination. If all oscillations are either to the right or to the left of the zero point, direct readings can be obtained. If the hand swings to both sides of the zero point, it is necessary to add the readings thus obtained to determine the oscillometric index. In other words, if the hand swings from 2 on the left side to 3 on the right, the oscillometric index is 5. Likewise, if the indicating needle swings from 2 on the left side to 7 on the right side, by subtraction an index of 5 is obtained. Similarly, if the needle swings from 2 on the left side to 7 on the left side, the oscillometric index is 5. An irregular pulsation will, of course, give irregular readings. In such a case, the maximum oscillation is recorded.

*Interpretation of Oscillometric Readings.*—It must be understood that the significance of oscillometric readings for diagnostic purposes lies not so much in the individual values obtained as in the comparison of readings between opposite extremities at the same level. In other words, if the oscillometric index at one ankle is 5 and at the other, at the same level, it is 2, this is evidence of abnormal arterial circulation in the extremity with the lesser reading. On the other hand, readings of 2 in each extremity at the same level may be normal for that particular individual. The normal range of readings in the extremities is as follows: just above the ankle, 1 to 10; calf, 3 to 10; popliteal region, 3 to 10; thigh, 4 to 20. In the upper extremities, at the wrist, 1 to 10; brachial region, 2 to 10; axillary region, 4 to 10. Again, it must be emphasized that this range is very wide and varies considerably with different individuals. It is only by comparing corresponding levels of the extremities that conclusions can be drawn concerning the patency of the arteries.

#### SUMMARY

Palpation of the dorsalis pedis pulse alone is an inadequate method of diagnosing organic arterial obstruction in the extremities. The test for plantar ischemia should be an essential part of every physical examination. A new simplified inexpensive oscillometer is described.

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## SENSITIZATION INDUCED BY TETANUS TOXOID, ALUM PRECIPITATED

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THE recent publication of Cooke and his associates<sup>1</sup> has served to center attention on the problem of sensitization induced by immunization with tetanus toxoid. In their very interesting study, Cooke and co-workers found that allergic reactions occur particularly after the second injection of alum-precipitated toxoid due to sensitization induced mainly by the peptones of the Berna or Witte type present in the toxoid. They also showed that sensitization to the bacterial products of *Cl. tetani* may take place.

Because of the importance of Cooke's observations we deemed it advisable to investigate this problem to determine (1) the frequency of sensitization as proved by skin tests; (2) the incidence of clinical evidence of sensitization; and (3) the possible existence of any correlation between (1) and (2).

Sixty-six patients that had been, or were undergoing, active immunization against tetanus were subjected to skin tests with two lots of materials. Lot A was prepared by a commercial biological house that employs Berna peptone in the manufacture of its tetanus toxoid, and lot B was supplied by a house that uses Difco proteose in its toxoid. All dilutions of lot A materials were made with Glenney's buffered salt solution. Lot B materials were diluted with sterile physiologic saline solution.

The following testing solutions were used: (1) tetanus toxoid A diluted 1:100 and 1:10; (2) Berna peptone A diluted to contain 0.01 and 0.10 mg. of nitrogen per cubic centimeter; (3) veal broth A diluted 1:10; (4) finished tetanus bouillon A diluted 1:100 and 1:10 (this preparation was a veal Berna peptone broth that had been subjected to all the manufacturing steps employed in the production of tetanus toxoid A, except that it had not been inoculated with tetanus bacilli); (5) tetanus toxoid B diluted 1:100 and 1:10; (6) Difco proteose B diluted to contain 0.01 mg. of nitrogen per cubic centimeter; and (7) tetanus media B diluted 1:100 and 1:10.

All persons included in this study had been treated with tetanus toxoid alum-precipitated A. A few also received intranasal instillations of tetanus toxoid topagen A. They may be classified in eight groups according to their immunization history.

Group 1 consisted of 7 patients on whom skin tests were performed two days before and thirty-two days after they had received the first injection of 1.0 c.e. of tetanus toxoid, alum precipitated.

Group 2 consisted of 29 persons on whom skin tests were done seventy-eight days after they had received the first 1.0 c.e. injection of alum toxoid.

Group 3 consisted of 4 persons on whom skin tests were done 207 days after they had received the first injection of 1.0 c.c. of alum toxoid. Thirty-two days later they were injected with a second dose of alum toxoid. Recheck skin tests were done thirty-two days after the latter was given.

Group 4 consisted of 3 patients (Nos. 19, 20, and 36) on whom skin tests were done 1,045, 1,544, and 1,479 days, respectively (about three to four and one-half years), after they had each received two doses of alum-precipitated toxoid. A third dose of 1.0 c.c. of alum toxoid was administered to two of them (Nos. 20, 36) fourteen days and one day, respectively, after the first skin tests were done. Skin retests were performed thirty-five and twenty-five days, respectively, after the third injection.

Group 5 consisted of 6 persons. Four of them, patients 17, 18, 30, and 35, had received three injections of alum toxoid (0.5 c.c., 0.5 c.c. and 1.0 c.c.) before the first skin tests were performed. These tests were made 766 to 809 days (about twenty-six months) after the third dose of toxoid was given. A fourth injection of 1.0 c.c. of alum toxoid was administered to the 4 patients fourteen, ten, two, and one day, respectively, after the first skin tests were performed. The skin tests were repeated twenty-eight, thirty-two, thirty-two, and twenty-five days, respectively, after the fourth injection of toxoid. The fifth member of group 5, patient 22, was tested 792 days (about two years) after he had received three 1.0 c.c. injections of alum toxoid. Twelve days later he was given a fourth injection of 1.0 c.c. of toxoid. Skin retests were done thirty-five days later. The last member of the group, patient 21, had received four injections of alum toxoid (0.5 c.c. each) prior to skin testing. Thirty-seven months had elapsed between the last dose of toxoid and the first skin tests. Fourteen days after the tests were performed, this person received a fifth injection of 1.0 c.c. of toxoid. The skin tests were repeated thirty-five days later.

Group 6 consisted of 5 patients that had been immunized by the combined subcutaneous and intranasal routes.<sup>2</sup> Prior to the two daily intranasal instillations of tetanus toxoid topagen, patient 31 had received two injections (1.0 c.c. each); patient 34, 3 injections (0.5 c.c. each); patient 37, 5 injections (1.0 c.c., 0.5 c.c., 0.5 c.c., 1.0 c.c., 1.0 c.c.); patient 38, 3 injections (1.0 c.c. each); and patient 39, 3 injections (0.5 c.c., 0.5 c.c., 1.0 c.c.) of alum-precipitated toxoid. Skin tests were performed on the entire group 210 to 263 days after the last toxoid topagen instillation in the nose. One to four days later they all received an additional injection of 1.0 c.c. of alum toxoid. Skin retests were done thirty to forty-two days after this final injection.

Group 7 included 9 patients, 4 of whom had received two courses of three daily intranasal instillations of tetanus toxoid topagen. Four others obtained the same treatment, except that the second course consisted of only two daily instillations. The ninth person received two courses of two daily intranasal topagen instillations. The entire group was skin tested eighty-four days after the last nasal treatment. Thirty-two days later they were all injected subcutaneously with 1.0 c.c. of alum toxoid. Skin retests were done thirty-two days after this injection.

Group 8 consisted of 3 persons, 2 of whom received a single course of three daily topagen instillations in the nose prior to skin testing, while the third

patient got an injection of 1.0 c.c. of alum toxoid previous to the course of nasal treatment. Thirty-two days after the first skin tests were done, all 3 received an injection of 1.0 c.c. of alum toxoid. Actually 240 and 146 days, respectively, had elapsed between the last intranasal instillation and the final injection of toxoid. The skin tests were rechecked thirty-two days after the last injection.

#### SKIN TESTS

About 0.02 c.c. of each testing solution was injected intraeutaneously, and the reactions were read at the end of fifteen minutes and twenty-four hours. The immediate reactions (fifteen minutes) were described as negative, doubtful, slight, moderate, and marked in accordance with the usual allergic practice.<sup>3</sup> In the instances where half-hour readings were made, the reactions did not materially differ from those recorded at the end of fifteen minutes. The delayed reactions consisted of local induration with papule formation, erythema of varying degrees and swelling. In many cases these reactions did not subside until forty-eight to seventy-two hours had elapsed.

The 7 persons tested before immunization showed negative reactions. Of 40 patients tested one to six and one-half months (largest number done at two and one-half months) after the first injection of 1.0 c.c. of tetanus toxoid-alum precipitated (groups 1, 2, and 3), 20 gave positive reactions of the immediate or delayed type to one or more of the testing solutions, while 20 were negative. The reactions varied in intensity from slight (+) to marked (++++). Two persons gave slight immediate reactions to veal broth A 1:10. One person gave a slight immediate reaction to tetanus media B 1:100, while 2 others gave a similar reaction to the 1:10 dilution of this material. None of these patients showed delayed reactions to either the veal broth A or the tetanus media B. Two gave slight immediate reactions to Berna peptone A, 0.01 mg. of nitrogen per cubic centimeter. One of them also showed a positive delayed response. One showed a moderate immediate as well as a delayed reaction to this peptone. Three persons gave positive delayed reactions though their immediate response was negative. Fifteen gave positive immediate reactions to Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter. They were recorded as one questionable slight (?), 13 slight, and one marked. Six of these patients also gave positive delayed reactions. One gave a positive delayed reaction to this peptone, although his immediate reaction was negative. Only 2 gave slight immediate reactions to the Difeo proteose B, 0.01 mg. of nitrogen per cubic centimeter. Nine persons gave positive immediate reactions to tetanus toxoid A, 1:10; in 3 they were read as questionable slight (?), in 5 they were slight, and in one the reaction was marked. Six of these also gave positive delayed reactions. Three with a doubtful ( $\pm$ ) immediate reaction gave a positive delayed response. Six gave a positive immediate reaction to tetanus toxoid B, 1:10. In three the reaction was slight, in 2 it was moderate, and in one it was marked. Three of these also gave positive delayed reactions. One gave a definite delayed reaction though his immediate response to the toxoid B was doubtful ( $\pm$ ). The reactions to the finished tetanus bouillon A were in agreement with those obtained with the veal broth A and Berna peptone A.

Three subjects of group 3 were skin-tested thirty-two days after the injection of the second dose of toxoid. In 2 there were no evidences of skin sensitivity. In the third patient only slight positive reactions occurred at the sites of the tetanus toxoid A and B, 1:10, although before the injection of the second dose this person had shown positive reactions to veal broth, Difco proteose, and Berna peptone as well. We cannot account for this discrepancy. In three members of group 4 the skin tests were performed about three to four years after the second dose of toxoid was injected. Patient 19 gave a slight reaction to toxoid B. Patient No. 20 showed slight immediate as well as delayed reactions to tetanus toxoids A and B. Patient 36 gave slight immediate reactions to toxoids A and B and Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter. The last two patients were retested thirty-five and twenty-five days, respectively, after a third injection of 1.0 c.c. of alum toxoid was given. Patient 20 then showed slight immediate reactions to toxoids A and B, and a marked reaction to Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter. Delayed reactions were also present. On the other hand, patient 36 showed immediate marked reactions to both toxoids A and B, and a slight + reaction to the Berna peptone but no delayed reactions.

When skin tests were done two to three years after three to four injections of alum toxoid (varying doses) had been given to the 6 members of group 5, we found that patient 18 showed slight reactions to tetanus toxoids A and B, and that patient 35 showed a slight reaction to toxoid B. The rest of the tests were negative. When retested about one month after the injection of an additional dose of alum toxoid, 3 members of this group, patients 18, 35, and 22, showed slight immediate reactions to tetanus toxoids A and B. Patient 18 also gave a questionable slight reaction to Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter and tetanus media B, 1:10. Also patient 30 showed a slight reaction to tetanus toxoid A, while patient 17 gave a similar response to tetanus toxoid B. The most interesting reactions were shown by patient 21. A marked immediate reaction was elicited by tetanus toxoid A and Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter, while a questionable slight response was brought forth by tetanus toxoid B. Veal broth A and Difco proteose B were negative, and tetanus media B gave a slight reaction. Intense delayed reactions occurred at the sites of toxoid A and finished tetanus bouillon A. Similarly, patients 31 and 37 (group 6) showed slight reactions to toxoids A and B. Patient 37 also gave slight reactions to Berna peptone A and veal broth A. After the injection of an additional dose of alum toxoid, patient 31 also showed a slight reaction to Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter. Also patients 34 and 38 (group 6) who before the final injection of toxoid had shown negative skin reactions, now gave positive reactions to toxoids A and B. Actually patient 38 showed a moderate reaction to toxoid B. Both of these patients showed delayed reactions. Patient 39 (group 6) remained negative, notwithstanding the four injections of alum toxoid and the three intranasal instillations of toxoid topagen that she had received. Skin tests performed on patients of groups 7 and 8 that received intranasal instillations of toxoid topagen were completely negative except in one. Retests done after the injection of 1.0 c.c. of alum toxoid were also negative. The exception, patient 6, who showed

slight reactions to toxoids A and B, had received an injection of alum toxoid prior to the topagen instillations. The absence of skin reactions in these two groups may be purely accidental, since none of the patients showed any evidence of sensitization even after the injection of 1.0 c.c. of alum toxoid.\*

#### DISCUSSION

Ample evidence is provided by this study in support of the belief that sensitization is an extremely individualized process. Thus, while patient 65 (group 2) gave marked reactions after one single dose of alum toxoid, patient 39 (group 6) showed negative skin reactions after he had received four injections of toxoid and two daily intranasal instillations of toxoid topagen. It is also apparent that sensitization as demonstrated by skin tests occurs rather frequently following the injection of tetanus toxoid alum precipitated.

Particularly worthy of note are the reactions obtained in several patients. Thus patient 25 (group I) showed a slight reaction to tetanus toxoid A, 1:10, and a marked reaction to tetanus toxoid B, 1:10, but was negative to the rest of the test solutions. Positive delayed reactions were produced by both toxoids as well as Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter. These results were confirmed on two subsequent retests. Patient 58 (group 2) showed a slight reaction to Berna peptone A and a moderate reaction to toxoid B, 1:10. The latter also gave a pronounced delayed reaction. The rest of the tests were negative. The same type of reaction to tetanus toxoid B was found in patient 36 (group 4) and in patient 38 (group 6).

These findings would indicate that sensitization to the toxoid protein or to bacterial proteins of *Cl. tetani* has occurred in these patients as a result of the injection of 1.0 c.c. of alum toxoid A. The difference in the skin reaction to toxoids A and B is probably due to differences in concentration of these proteins as found in the two toxoid preparations.

Patient 65 (group 2) gave a marked reaction to toxoid A, 1:10, and Berna peptone A, and a slight reaction to veal broth A and tetanus media B. He was negative to Difeo proteose B. Also patient 21 (group 5) showed marked reactions to toxoid A and Berna peptone A, and slight reactions to toxoid B and tetanus media B. These findings would indicate the existence of sensitization to Berna peptone. Confirmatory proof is to be found in the large group of patients that gave slight positive reactions to this protein. It would appear that Berna peptone when present in the alum toxoid used for immunization sensitizes patients more readily than the Difeo proteose. This is in agreement with Cooke's observation.

*Delayed Reactions.*—Patient 29 (group I) is of interest because she gave prominent delayed reactions to toxoids A and B and to Berna peptone A though the immediate reactions to these products were questionable slight positive. The peptone reaction was especially intense. These findings were confirmed on two subsequent rechecks. The mechanism of these delayed reactions is not clear. As expected this patient gave a negative passive transfer test.

\*It must be remembered that these patients did not develop any demonstrable antitoxin titer following the intranasal instillation of topagen. The latter is of value only as a repeat stimulus after a basic immunity has been established through the subcutaneous injection of one or preferably two doses of alum-precipitated toxoid.<sup>2</sup>

Similar delayed reactions occurred in a number of our patients. They consisted of either erythema of varying size and intensity or a bright red area of raised induration with a centrally placed papule. Localized swelling of the soft tissues around the site of injection was usually present. Occasionally there was some itching. These delayed reactions began to fade after twenty-four hours and disappeared completely at the end of forty-eight to seventy-two hours. Parish and Oakley<sup>7</sup> also noted "late" reactions "like those occurring after staphylococcus toxoid," but they were at their maximum about two hours after the injection of the testing products and faded completely during the next three hours. Cowles<sup>9</sup> also noted in one immunized person a curious delayed reaction that came on in twenty-four hours.

At present we are unable to evaluate the significance of these delayed reactions. In many cases, an immediate response to the testing solutions occurred as well. The immediate type of reaction was proved by Cooke and associates to be mediated through sensitizing antibodies that circulate freely in the blood and which can be passively transferred to the normal skin (Prausnitz-Küstner phenomenon). We performed passive transfer tests with the serum of patients 21 (group 5), 25, and 29 (group 1) in accordance with the usual technique.<sup>3</sup> Each serum was injected into the skin of three normal nonimmunized persons (0.1 c.c. in each site). Two of the three recipients gave positive reactions at the sites sensitized with the serum of patient 21 when tested with tetanus toxoid B, 1:100, Berna peptone A, 0.10 mg. of nitrogen per cubic centimeter, and tetanus toxoid A, 1:100. All three recipients gave negative reactions at the sites sensitized with the sera of patients 25 and 29.

*Duration of Skin Sensitivity.*—In the presence of positive skin tests the question arises as to their duration. Cooke reported that one person lost his marked sensitivity within six months. However, another person showed a marked urticarial wheal when retested with tetanus toxoid 1:10 eight months after the second dose had been injected. Passive transfer test with the serum then obtained was weakly positive. We retested patients 21 (group 5), 25, 29 (group 1), 58, and 65 (group 2) six months after the last tests described in the text were performed. At this time patient 21 showed no change in the immediate reactions, but there were no delayed reactions. Patient 25 showed negative immediate reactions to toxoid A and to Berna peptone, 0.10 mg. of nitrogen per cubic centimeter and a moderate reaction to toxoid B, 1:10. Similarly, there was a definite decrease in the size of the delayed responses which occurred at the sites of toxoid A and B and Berna peptone A. Patient 29 showed no change in the reaction to toxoid A, but there was a definite increase in the response to Berna peptone A and to toxoid B. The latter gave a marked reaction. Again the delayed reactions in this patient were very prominent, especially to the Berna peptone. Patients 58 and 65 showed no change in their reactions. On the other hand, the results obtained in the comparative skin tests of several patients suggest that skin sensitization may disappear with time. Thus the absence of positive skin reactions in patient 21 (group 5), when he was tested three years after he had received four injections of alum toxoid as contrasted to the marked reactions obtained one month after the fifth injection of toxoid, can be best explained on the basis that we are dealing here with reactiva-



tion of a sensitivity that had decreased with time. This explanation is more likely to be true than the alternate assumption that the first four injections of toxoid did not sensitize this person while the fifth one did.

*Significance of Positive Skin Tests.*—It is evident from our studies that a high percentage of persons immunized with tetanus toxoid alum precipitated that contains Berna peptone develop positive skin tests to the toxoid and its component constituents. But as in other fields of allergy, these skin tests to be significant must be correlated and associated with definite evidences of specific clinical sensitivity.

At the outset we were struck by the disparity between the high incidence of positive skin tests and the marked scarcity in our experience of untoward systemic reactions following the injection of two or more doses of tetanus toxoid alum precipitated. A review of the literature reveals a similar paucity of reports of allergic reactions induced by tetanus toxoid. In France where many thousands of men have been injected with three or more doses of Ramon's anatoxin, the procedure was reported as harmless, not being followed by any local or general reaction.<sup>4</sup> In England Boyd<sup>5</sup> also noted the absence of reactions. Recently two very interesting reports have appeared in the *British Medical Journal*. Whittingham<sup>6</sup> reported two cases of anaphylaxis (nonfatal) that occurred among the 61,042 members of the Royal Air Force who had been immunized with two 1.0 c.e. doses of plain toxoid given about six weeks apart. The toxoid contained Witte peptone, which was held responsible for the severe peptone shock, since both patients gave positive skin tests to this protein. Whittingham summarized his experience as follows:

| TOTAL NO. PERSONS<br>INOCULATED  | ACUTE ANAPHYLACTIC<br>REACTION<br>A | DEFINITE CONSTITU-<br>TIONAL SYMPTOMS<br>B | LOCAL REACTIONS<br>ONLY<br>C |
|--|-------------------------------------|--|------------------------------|
| 61,042   | 2 patients<br>(0.003%)              | 12 patients<br>(0.02%)                     | 651 patients<br>(1.06%)      |
| A. Anaphylactic shock.   |                                     |  |                              |
| B. Influenza-like aching of head and body. Slight pyrexia. Seven of these persons had urticaria of limbs and body.   |                                     |  |                              |
| C. Brawny swelling and pain for twelve to twenty-four hours. Twenty-two patients had urticarial swelling and itching around site of inoculation for a few hours. |                                     |  |                              |

Parish and Oakley<sup>7</sup> also reported one patient with anaphylaxis after the second injection among over 300 members of the staff of the Wellcome Laboratories that received two injections each of 1 c.e. of plain toxoid. The severe reaction was controlled by epinephrine. This patient had received two courses of staphylococcus toxoid, which has a high content of Witte peptone, two years and one year previous to the administration of the tetanus toxoid. This patient, as well as a number of persons who had received two injections of tetanus toxoid without showing any anaphylactic symptoms, gave marked skin reactions to Witte peptone and to peptic digests of beef, pork, and horse fibrin. Parish and Oakley stated that their observations "throw considerable doubt on the value of scratch and intradermal tests as usually interpreted on the basis of early readings." "Late" reactions appeared to be important. Because the patient in question was the only subject who developed mild anaphylactic symptoms soon after the 1:1,000 dilutions of Witte peptone and two other beef fibrin digests

had been injected intraeutaneously, the authors felt that Witte peptone might have been the substance in tetanus toxoid to which the patient had become sensitive. They concluded that allergic phenomena, though alarming, appear to be rare and are readily controlled by the injection of adrenaline. It is important to remember that these two large series of cases were immunized with tetanus toxoid plain, which contains larger amounts of peptone than alum-precipitated toxoids. On the other hand, the latter may act as a better sensitizing agent.

In the United States tetanus toxoid alum precipitated has been used almost exclusively. In their report, Cooke and co-workers refer to two patients with urticaria besides our own case. The two cases were encountered by Hall after the injection of a second dose of toxoid (not alum precipitated). Since publication of Cooke's study, we have immunized 250 additional patients with two 1.0 c.c. doses of alum-precipitated toxoid A without a single systemic reaction. Three persons had severe local reactions which subsided in twenty-four to forty-eight hours. Because individual experiences are bound to be limited in scope, we have collected additional information through the kind cooperation of several workers in this field.<sup>9</sup> This collective experience with tetanus toxoid alum precipitated is summarized in Table I.

Over 1,700 patients have been immunized with two doses of alum toxoid. Many received three injections. Only two cases of urticaria have occurred. One of them resulted after a number of injections had been given to an allergic child for hyperimmunization. The very small number of reactions in this large group of patients is in agreement with the experience of Whittingham<sup>6</sup> and is in sharp contrast with Cooke's "belief that reactions occur more often than the present reports indicate." In his series of about 25 cases, Cooke and collaborators found four instances of induced sensitization with but one reaction of serious proportion. Actually there was only one patient with urticaria, while a second subject gave a very marked swelling with itching at the site of the second injection that subsided in twenty-four hours. In an addendum, Cooke stated that two additional cases of delayed urticaria were encountered. In one it occurred after the second dose; in the other, after the third injection. Neither case showed any significant skin test reaction to the toxoid (1:10) before or after the therapeutic injection.

From the data we have gathered it appears that following immunization with tetanus toxoid alum precipitated skin sensitization occurs much more often than clinical evidences of allergy. Actually the latter are a rather rare occurrence easily controlled by epinephrine hydrochloride. Moreover, an untoward allergic reaction may occur in the absence of any significant skin reaction (Cooke's two cases). Conversely, it does not follow that the presence of a positive skin test contraindicates the administration of tetanus toxoid. To insist upon a negative skin test before the injection of alum toxoid is given is not warranted by our experience. For example, among our own cases after significant positive skin reactions were obtained in two patients (Nos. 58 and 65, group 2), three months after the first dose of alum toxoid A had been given, they received a second dose without any mishap. Six months later these two patients were retested and again after obtaining marked skin reactions to tetanus toxoids A and B and Berna peptone A, a third dose of alum toxoid A was in-

TABLE I  
DATA OF REACTIONS IN 1,700 PERSONS AFTER THE INJECTION OF TWO OR MORE DOSES OF TETANUS TOXOID ALUM PRECIPITATED (COLLECTED FROM THE LITERATURE AND PERSONAL COMMUNICATIONS FROM INVESTIGATORS IN THE UNITED STATES)

| AUTHOR                    | NO. OF PATIENTS | NO. OF INJECTIONS                              | ALUM-<br>PRECIPITAT-<br>ED TOXOID             | REACTIONS   | SKIN TESTS   |
|---------------------------|-----------------|--|---|---|--|
| A. McBryde*               | 125<br>children | 2  | B and D                                       | No allergic reaction. Occasional moderate<br>severe local reaction  | Not done   |
| C. P. Brown*              | 100             | 50 had 2<br>50 had 3                           | C   | None†   | Not done   |
| M. Fernan-Nunez*          | 500             | 2  | D and C                                       | None (remarkably free of reaction)  | Not done   |
| P. B. Cowles*             | 125             | Most had 2<br>Good many 3                      | C and B                                       | One student developed about 2° fever with<br>headache and malaise after second in-<br>jection. Subsided during night  | 20† had skin tests done, with<br>plain tetanus toxoid (bac-<br>topeptone) 1:500 saline<br>dilution |
| R. Layden and W. W. Halls | 199             | 3  | B   | None  | Not done   |
| H. Gold                   | 500             | Most had 2<br>Many had 3 or<br>more injections | 470 had A<br>10 had B<br>10 had C<br>10 had D | One patient had generalized urticaria after<br>second dose. Controlled by adrenaline  | 66 had skin tests done   |
| A. F. Coen*               | 180             | 2  | B   | 118 had local reactions<br>17 had local and general reactions, the<br>latter consisting of headache, anorexia,<br>malaise, and occasional nausea<br>5 had only general reaction<br>No allergic reactions were encountered | Not done   |
| R. M. Kempton*            | 8               | 2  | C   | None  | Not done   |

Tetanus toxoid alum precipitated A had Berna peptone.  
Tetanus toxoid alum precipitated B had Difco peptose.  
Tetanus toxoid alum precipitated C had Martin's peptone.  
Tetanus toxoid alum precipitated D had Berna peptone.  
\*Personal communication.  
†A physician reported that an allergic child had a constitutional reaction consisting chiefly of urticaria which occurred after a number of In-  
jections had been given for hyperimmunization. (For possible use as blood donor.)  
‡Five-hundredths cubic centimeter was injected intracutaneously. Eight weeks after the second injection 3 students gave positive reactions and one  
in an hour or so. Of these positives one was a most pronounced had an indurated wheal of 1 sq. cm. in an erythematous zone of 1 sq. cm. vanishing  
made on this person with consistent results. Seven months after these tests, one of the mild reactors received 0.6 ml. of plain toxoid intradermally.  
Within fifteen minutes there developed an indurated zone about 3.5 cm. in diameter in an erythematous area 7.5 by 15 cm. The wheal persisted for  
eight hours while the erythema was visible the next day. When the erythema and induration were slight and transient -1 cm. and lasting only for an  
hour or so, injections were given without mishap. There was no real reason, other than an excess of caution, for failing to give all reactors the  
usual recall injection.

5. Little practical value can be expected from routine skin testing before the injection of tetanus toxoid. This would only serve to render cumbersome an otherwise simple and effective method of immunization against tetanus. As in any other immunizing procedure, the occasional occurrence of an allergic reaction should be kept in mind and epinephrine hydrochloride, 1:1,000, should always be available.

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314 E. BROAD STREET

# LOCALIZATION AND CONCENTRATION OF STAPHYLOCOCCUS ANTITOXIN IN AREAS OF RABBIT'S SKIN\*

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IN RECENT publications it was shown that staphylococcus antitoxin following an intravenous injection localizes and concentrates in areas of rabbit's skin where xylol is applied.<sup>1, 2</sup> Foreign serum and antibodies have been demonstrated by others in areas of inflammation. Menkin<sup>3</sup> showed the presence of horse serum following an intravenous injection in areas of inflammation by extracting the tissues in saline and using the precipitation reaction. Fox<sup>4</sup> used a similar technique to demonstrate the localization and concentration of agglutinins in areas of inflammation.

The present paper is a report of some observations on the failure of dermal necrosis to occur in the rabbit when staphylococcus toxin is injected intradermally into areas where xylol has been applied in animals previously given staphylococcus antitoxin intravenously.

## METHODS AND MATERIAL

Normal rabbits are used. They are carefully shaven twenty-four hours or longer before the experiment is begun. An area, approximately 4 by 6 cm., is outlined with India ink on one side of each rabbit. Five cubic centimeters of staphylococcus antitoxin is given intravenously in the marginal vein of the ear. Xylol, on a cotton applicator, is applied to the demarcated area of skin immediately after the injection of the antitoxin. One tenth cubic centimeter of staphylococcus toxin is injected intradermally in the xylol-treated and untreated skin of the same rabbit at intervals varying from immediately to nine days following the injection of the antitoxin.

In the control experiment xylol is applied similarly to the skin of six rabbits, and staphylococcus toxin is injected intradermally in each of two rabbits at the following intervals after the application of the irritant: five, twenty-four, and forty-eight hours.

The presence or absence of skin necrosis at the site of the injection of the toxin in the xylol-treated areas of skin is considered indicative of either the absence or the presence of antitoxin. The control for each experiment is the presence of necrosis at the site where the toxin is injected in the normal skin.

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A portion of this work was done in the Department of Pathology, Vanderbilt University, Nashville, Tenn.

The staphylococcus antitoxin and the staphylococcus toxin were supplied by Lederle Laboratories.

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A marked variation in the size of the necrosis in each rabbit is also considered significant. Slight variations in the size of the necrosis may be observed when a series of injections of equal size are made in a single rabbit.

TABLE I

NECROSIS PRODUCED BY STAPHYLOCOCCUS TOXIN IN XYLOL-TREATED AND UNTREATED AREAS OF SKIN IN RABBITS GIVEN STAPHYLOCOCCUS ANTITOXIN INTRAVENOUSLY

| RABBIT | TIME BETWEEN APPLICATION OF XYLOL AND INJECTION OF TOXIN | AREAS OF NECROSIS AFTER 24 HOURS |                    |
|--------|--|----------------------------------|--------------------|
|        |  | AREA WITH XYLOL                  | AREA WITHOUT XYLOL |
| 346    | Immediate  | 0.4 by 0.5 cm.                   | 0.4 by 0.5 cm.     |
| 347    | Immediate  | 1.0 by 1.2 cm.                   | 0.5 by 0.6 cm.     |
| 399    | 2 hours  | No necrosis                      | 1.5 by 2.0 cm.     |
| 400    | 2 hours  | No necrosis                      | 1.0 by 1.5 cm.     |
| 363    | 3 hours, 15 minutes                                      | 0.15 by 0.15 cm.                 | 1.2 by 1.2 cm.     |
| 364    | 3 hours, 15 minutes                                      | No necrosis                      | 0.6 by 1.5 cm.     |
| 369    | 24 hours   | No necrosis                      | 1.5 by 2.5 cm.     |
| 370    | 24 hours   | No necrosis                      | 1.5 by 1.5 cm.     |
| 376    | 44 hours   | 0.15 by 0.15 cm.                 | 1.0 by 1.5 cm.     |
| 377    | 44 hours   | 0.2 by 0.2 cm.                   | 1.5 by 2.5 cm.     |
| 378    | 5 days   | 0.7 by 0.8 cm.                   | 0.8 by 1.4 cm.     |
| 379    | 5 days   | 0.1 by 0.1 cm.                   | 0.2 by 2.0 cm.     |
| 380    | 9 days   | 1.5 by 1.5 cm.                   | 1.0 by 2.0 cm.     |
| 381    | 9 days   | 2.0 by 2.0 cm.                   | 1.5 by 2.0 cm.     |
| 367*   | 5 hours  | 2.0 by 9.0 cm.                   | 2.5 by 6.0 cm.     |
| 371*   | 5 hours  | 2.0 by 8.0 cm.                   | 2.0 by 6.0 cm.     |
| 393*   | 24 hours   | 1.5 by 3.0 cm.                   | 3.0 by 6.0 cm.     |
| 391*   | 24 hours   | 1.5 by 5.0 cm.                   | 5.0 by 6.0 cm.     |
| 388*   | 48 hours   | 2.0 by 5.0 cm.                   | 3.0 by 6.5 cm.     |
| 389*   | 48 hours   | 1.5 by 6.0 cm.                   | 2.5 by 6.0 cm.     |

\*These rabbits received no antitoxin.

Rabbits given 5 c.c. of staphylococcus antitoxin.

All rabbits received 0.1 c.c. of toxin intradermally.

#### EXPERIMENTAL

The xylol-treated areas of skin become hyperemic and edematous within a period of minutes and continue to show macroscopic evidence of inflammation for approximately five days.

Staphylococcus toxin when injected intradermally produces necrosis in the normal skin within twenty-four hours. Table I shows the size of the necrotic areas in the xylol-treated and untreated skin when staphylococcus toxin is injected intradermally twenty-four hours previously. Furthermore, these data in this table show that all the rabbits in which xylol is applied to the skin and no antitoxin is given intravenously have areas of necrosis in the xylol-treated areas of skin approximately the same size as those in the untreated skin. There is also no significant difference in the size of the areas of necrosis in the treated and untreated areas of skin in those rabbits in which the toxin is injected intradermally immediately following the intravenous injection of the antitoxin.

The areas of necrosis in the normal skin are always smaller in the rabbits given antitoxin than they are in those rabbits given no antitoxin.

When the interval between the intravenous injection of the antitoxin and the intradermal injection of the toxin is two hours, complete inhibition of necrosis occurs in the areas of skin where the xylol is applied. There is also marked inhibition of skin necrosis produced by staphylococcus toxin when

xylo is applied from forty-four hours to five days following the injection of the antitoxin. After nine days the extent of the necrosis produced by the toxin in the area where xylo is applied is the same size as that which occurs in the untreated skin of the same rabbit.

#### DISCUSSION

The experiment shows that there is an absence of necrosis following an intradermal injection of staphylococcus toxin in those areas of skin where xylo is applied immediately after an intravenous injection of staphylococcus antitoxin when the toxin is given from two to forty-four hours later. This inhibition of local necrosis apparently results primarily from the neutralization of the toxin by the antitoxin. Staphylococcus antitoxin apparently accumulates in a greater quantity in the inflamed tissue than in the normal skin, as shown by the absence of necrosis in the xylo-treated areas. The presence and absence of necrosis in the skin of the same rabbit is a good control and eliminates the necessity of determining the variation in the normal staphylococcus antitoxin titer of each animal.

The size of the skin necrosis produced by staphylococcus toxin in the areas where xylo is applied apparently may be slightly influenced by factors other than antitoxin. This is shown by the slight variation in the size of the areas of necrosis produced by toxin in the rabbits treated with xylo twenty-four to forty-eight hours previously. Some of the staphylococcus toxin may be removed from the site of inoculation in the xylo-treated areas of skin as a result of an increase in the flow of lymph and blood. It is essential to inject the same quantity of toxin into the xylo-treated skin in rabbits given antitoxin as is necessary to produce definite areas of necrosis in the xylo-treated areas of the skin in the same animal without the addition of antitoxin.

It is interesting that an interval of approximately two hours is necessary before a sufficient quantity of antitoxin accumulates where xylo is applied to neutralize completely the amount of toxin used in this experiment. The antitoxic effect, sufficient to inhibit necrosis, remains in the tissue for a limited time only. This is shown by the presence of necrosis where xylo is applied, and the antitoxin is given five days before the intradermal injection of the toxin. Approximately equal size areas of necrosis occur in the xylo-treated and untreated skin when staphylococcus toxin is injected nine days after the xylo is applied locally and the antitoxin is given intravenously.

It would appear most likely that staphylococcus antitoxin localizes and concentrates in areas of tissue as a result of the same mechanism which permits colloidal dyes to localize and to concentrate in areas of inflammation. Staphylococcus antitoxin like trypan blue fails to localize and to concentrate in areas of inflammation when the antitoxin is injected twenty-four hours following the local application of xylo.<sup>1</sup>

The concentration of antibodies in areas of inflammation may produce a local condition unfavorable for the development of an infectious process. Recently such a condition was thought to occur in mice passively immunized with staphylococcus antitoxin when they were given a subcutaneous injection of broth culture of staphylococci. There was a marked increase in the number of

leucocytes in the area of inflammation in the immune animals as compared with the number of leucocytes in the normal.<sup>5</sup>

It is conceivable that antibodies other than antitoxin may localize and concentrate in areas of inflammation provided there is an increase in capillary permeability. The favorable results obtained clinically by applying counter-irritants to local areas of inflammation produced by bacteria may be influenced by the localization of antibodies in the areas as a result of a local increase in capillary permeability.

It may be said that diphtheria antitoxin localizes and concentrates in areas of inflammation produced by the local application of xylo in the rabbit in a manner similar to that of staphylococcus antitoxin. No attempt has been made in these experiments to make a quantitative study on the localization of antitoxin in areas of inflammation.

#### SUMMARY

Staphylococcus antitoxin following an intravenous injection localizes and concentrates in areas of the rabbit's skin where the capillary permeability is increased through the local application of xylo. This localization of antitoxin is indicated by the development of a smaller area of necrosis where an intradermal injection of staphylococcus toxin is made into the xylo-treated skin. Antitoxin sufficient to inhibit necrosis localizes within approximately two hours and remains in the tissues for at least five days. The significance of the localization of antibodies in areas of inflammation and the use of counterirritants in the treatment of local infections are briefly discussed.

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# BACTERIEMIA PRODUCED BY AN AEROBIC, GRAM-NEGATIVE SPORULATING BACILLUS\*

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DURING 1938 our laboratory isolated an aerobic gram-negative, spore-forming bacillus from the blood cultures of five hospitalized patients. Because this motile organism on primary isolation apparently did not contain spores or ferment carbohydrates, it was thought at first to be a member of the *Alcaligenes* group. Further studies, however, revealed its ability to form spores. In addition, it was found to have no serologic relationship to *Alc. faecalis*.

TABLE I  
DATA REGARDING ISOLATION OF GRAM-NEGATIVE, SPORULATING BACILLUS

| PATIENT | DIAGNOSIS                                    | DATE     | BLOOD CULTURE |
|---------|--|----------|---------------|
| S       | Acute monocytic leukemia                     | 6/27/38  | Positive      |
|         |  | 7/ 1/38  | Positive      |
|         |  | 7/ 7/38  | Positive      |
|         |  | 7/12/38  | Negative      |
|         |  | 7/20/38  | Positive      |
| H       | Lymphocytic leukemia and pernicious anemia   | 7/16/38  | Positive      |
|         |  | 7/21/38  | Negative      |
|         |  | 7/23/38  | Negative      |
| C       | Pneumonitis, tracheo-bronchial lymphadenitis | 8/27/38  | Negative      |
|         |  | 8/30/38  | Negative      |
|         |  | 9/ 1/38  | Positive      |
|         |  | 9/ 8/38  | Positive      |
|         |  | 9/21/38  | Negative      |
| O       | Undulant fever                               | 9/14/38  | Positive      |
|         |  | 9/21/38  | Negative      |
|         |  | 10/ 7/38 | Negative      |
| B       | Pneumonia                                    | 11/18/38 | Positive      |
|         |  | 11/22/38 | Negative      |

A total of nine isolations were made over a period of five months (Table I). The blood cultures from three patients were positive once; from one other, twice; and from another, four times. In the last case the gram-negative bacillus was also isolated from the bone marrow and spleen at autopsy nine days after the last blood culture. Morphologically, biochemically, and tinctorially all five strains were identical, and there was but little doubt they comprised a single species. The consistency with which this bacillus was isolated in blood culture from one of these patients convinced us that we were not dealing with an ordinary contaminant, but with an actual inhabitant of the blood stream. Our purpose here is to submit the description of this bacillus and to report the results of serologic and pathogenic studies.

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## DESCRIPTION OF ORGANISM

*Morphology*

*Rods:* 0.7 to 0.8 by 3.4 to 4.5 microns. Tapered with rounded ends. Occur singly and in pairs, never in chains.

*Spores:* Ovoid, 0.9 to 1.2 by 1.2 to 1.7 microns. Subterminal to central swelling of the rod, clostridial appearance. Often naked. Formed early (within 18 hours) at 37° C.

*Motility:* Active and progressive in young cultures. Peritrichous flagella.

*Staining:* Gram-negative. Young cells sometimes variable.

*Cultural Characteristics*

*Colonies:* On extract agar young colonies are medium-sized circular, entire, and regular. They are transparent, colorless, and glossy. Older cultures may contain a variant which is grayish white and opaque. Both types are often present, giving appearance of mixed culture.

*Broth:* Profuse growth in extract broth with fine pellicle, heavy sediment, and slight turbidity. Streamers often appear in broth.

*Biochemical*

*Carbohydrates:* No action on any of the common sugars, or salicin, mannitol, inulin, rhamnose, cellobiose, sorbitol, glycerol, raffinose, inositol, or dextrin. Starch not hydrolyzed.

*Gelatin:* Liquefaction in from 6 to 8 days at 37° C.

*B. C. P. Milk:* No coagulation, no acid, becoming alkaline in four to five days. No peptonization in seven days.

Nitrates reduced to nitrites to ammonia; indol negative; slight H<sub>2</sub>S production in lead acetate agar. Grows in Koser's uric acid and sodium citrate media. No hemolysis on horse blood agar.

*Growth Requirements*

*Temperature:* Minimum temperature for growth, 25° C.; maximum, 50° to 55° C.; optimum, 37° to 42° C.

*Optimum pH:* Grows at a pH of 5.0 to 9.0. Optimum 6.0 to 8.0.

*Inhibition by Dyes:* Inhibited by dilutions of gentian violet not ordinarily affecting gram-negative organisms, namely, 1:200,000 to 1:400,000 on extract agar.

*Thermal Resistance of Spores:* Resists boiling water for 4 hours.

*Oxygen Requirements:* A strict obligate aerobe.

*Antigenic Structure.*—High-titered sera were produced by injecting rabbits with young, living, broth cultures. Cross agglutinations with flagellar antigens, as shown in Table II, indicate two serologic types. Strains H and S compose one type, while strains C, O, and B form a second. Reciprocal agglutination tests, using somatic antigens, showed a high degree of cross agglutination and suggested an O antigen common to both types.

TABLE II  
RECIPROCAL AGGLUTINATION TESTS

| FLAGELLAR<br>ANTIGEN | SERUM DILUTION TITER |       |      |       |
|----------------------|----------------------|-------|------|-------|
|                      | H                    | S     | C    | O     |
| H                    | 5120                 | 10240 | 160  | 20    |
| S                    | 5120                 | 10240 | 160  | 40    |
| C                    | 320                  | 80    | 5120 | 10240 |
| O                    | 320                  | 80    | 5120 | 10240 |
| B                    | 160                  | 160   | 2560 | 2560  |

*Pathogenicity.*—None of the isolations was pathogenic for rabbits. On two different occasions rabbits were injected intravenously with 1.0 c.c. doses of undiluted broth culture on alternate days for a period of two to three months. There were no ill effects. Blood cultures were repeatedly negative. Certain rabbits were sacrificed, and cultures made from the liver, spleen, and kidney. All were negative. Since two isolations (S and H) were from cases of leucemia, daily blood counts were carried out. No significant change in the blood picture was observed.

*Comparison With Dubos' Bacillus.*—Recently (1939, 1940) Dubos reported the isolation of a bactericidal substance from a soil bacillus (B. G.) which, when first described, appeared to resemble our organism. Morphologically and biochemically they are somewhat similar, but they differ in that Dubos' organism hemolyzes blood and produces spores of low heat resistance. There is no serologic relationship.

One strain of each agglutinative type was fractionated by Dubos' method for obtaining gramicidin. At the same time a search was made for a similar bactericidal substance obtained by Hoogerheide (1940) from members of the *B. subtilis* group. All attempts were unsuccessful.

#### DISCUSSION

An attempt to classify this gram-negative, spore-bearing bacillus has met with little success. *Bergey's Manual* (1939) contains numerous species of the genus *Bacillus* which, in many respects, resemble our organism. Nevertheless, of the species described in sufficient detail for accurate comparison, every one is different from the organism being reported in some major morphologic or biochemical characteristic. We believe, however, that an attempt to create a new species would be unwarranted without extensive serologic investigation.

The source and significance of this bacillus is open to conjecture. There is no evidence that it acts as a primary, or even a secondary, agent of disease. Its presence in the blood stream seemed to bear no relationship to the condition of the patients. No evidence was obtained that the number of organisms in the circulating blood at any one time was high. In addition, of the two sera tested (S and H) neither one contained specific antibodies. The highly resistant nature of the spores suggested that they might be found in saline, or on syringes and needles due to faulty sterilization. In this way, their presence in the blood stream could be explained.

Examination of several lots of glucose solution, saline, and syringes, however, did not yield this organism. The intestinal tract has been considered as a possible source. Nevertheless, cultures from stools of normal individuals were

consistently negative, although from one stool a spore-bearing bacillus of similar morphology was isolated. Each patient whose blood yielded this organism appeared to have lowered resistance. This may account for the survival and possible multiplication in the blood stream of an avirulent spore-former.

Although the literature contains a number of reports of the isolation of *Alc. faecalis* from blood (Andrieu and others, 1936), not all give an adequate description of the organism (Brown and Stewart, 1935; McIntyre, 1936; Goldberg, 1938). It is conceivable that in some of these instances the organism was actually this gram-negative spore-bearer, but due to the absence of spores on primary isolation from the blood stream it was mistaken for *Alc. faecalis*.

#### SUMMARY

1. An unidentified gram-negative, spore-bearing bacillus was isolated from the blood stream of five different patients.
2. From one individual the organism was isolated four times, from another twice, indicating a state of bacteriemia rather than external contamination.
3. The morphologic, biochemical, and serologic characteristics are presented.
4. The source and significance are discussed.
5. Any relationship to the B. G. organism of Dubos was ruled out. Search for a bactericidal substance by the methods of Dubos and of Hoogerheide was unsuccessful.

The generous assistance of Miss Frances Tuerffs in carrying out the pathogenicity studies is acknowledged with gratitude.

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# SEPARATION OF THE COAGULANT FROM THE TOXIC PRINCIPLES OF THE VENOM OF THE AUSTRALIAN TIGER SNAKE (NOTECHIS SCUTATUS)\*

WITH REMARKS ON THE MODE OF ACTION OF THE COAGULANT

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THE venom of the Australian tiger snake (*Notechis scutatus*) is one of the most powerful snake poisons known. Injected subcutaneously, it is more potent per milligram than any other known snake venom.<sup>1</sup> Death is attributable to the action of an extremely active neurotoxin.<sup>2</sup> Tiger snake venom also contains a powerful coagulant substance. Very minute amounts can rapidly clot oxalated or citrated blood in vitro or cause intravascular coagulation in vivo on intravenous injection.<sup>3, 4</sup> Only the venoms of the South American vipers, the jararaca (*Bothrops jararaca*) and the fer-de-lance (*Bothrops atrox*), and the Australian black snake (*Pseudechis porphyriacus*) are comparable in coagulating power. This property of tiger snake venom has been utilized clinically to arrest severe capillary hemorrhage by topical application.<sup>4</sup> The coagulant action of snake venoms has led also to their introduction into the study of problems concerned in blood coagulability, and has served to increase our knowledge of this process.<sup>5, 6</sup> It has been found that the coagulant factor of tiger snake venom produces its effect in the same way as does thrombokinase, except that the presence of calcium is not required; in other words, it can in itself activate prothrombin to thrombin.<sup>5, 6</sup>

A great deal of work has been done in attempting to separate the coagulant from the neurotoxic factor of tiger snake venom, but hitherto only the toxic factor has been successfully separated.<sup>7, 8</sup> In the present investigation a simple method has been devised to separate the coagulant from the toxic factor of tiger snake venom. It is based on the action of hydrochloric acid upon the venom.

A 1:1,000 solution of powdered tiger snake venom in physiologic salt solution (0.85 per cent NaCl) is made. The solution is filtered through ordinary filter paper to clear it of any debris. Hydrochloric acid, in strength of 1.4 normal or 5 per cent, is added in equal volume. After several minutes an opalescence is noted, which at the end of one-half to one hour forms a precipitate. The resulting mixture is allowed to stand overnight in a refrigerator. The next day the mixture is centrifuged at 2,500 to 3,000 r.p.m. for twenty minutes in order to get a well-packed layer of the precipitate and to effect complete separa-

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tion from the supernatant fluid. The supernatant fluid, which contains the toxin, is carefully decanted or pipetted so as not to disturb any of the sediment in which the clotting factor is present in an inactive state. An extremely small particle of sediment carried over into the supernatant solution will cause the fluid on neutralization to exhibit a significant degree of clotting power. The fluid is, therefore, filtered through a special grade of filter paper (Whatman No. 42), or through a No. 4 Jena fritted glass crucible. The sediment (precipitate) is broken up and dissolved in distilled water. The volume is made up to that of the original venom solution. It is then reprecipitated with 1.4 normal acid. After waiting one-half to one hour the precipitate and the supernatant are separated as before. The processes of precipitation and separation are again repeated. The three precipitations are necessary to free the coagulant present in the sediment from the small amount of absorbed toxin.

The sediment obtained after the third precipitation is separated by centrifuging as before, and then broken up and dissolved in a volume of distilled water equal to half the volume of the original venom solution. It is now carefully neutralized to a pH of 7.0 to 7.2, or to the pH of the original venom solution. The volume is then brought up to that of the original venom solution with distilled water and is ready for testing. It is essential that the resulting solution shall be water clear. If the solution shows any opalescence it will be much less potent in coagulant activity than a clear solution. During the process of neutralization a heavy precipitation occurs at about a pH 6.5, which may be the isoelectric point of some substance in this preparation. It is important to pass over this point quickly; otherwise a final clear solution may not be obtained.

The three supernatant solutions after filtration are neutralized to pH 7.2, and tested for clotting action. The volumes and any additions are recorded for calculating the dilution of toxin. They are then tested for possible clotting action upon fresh citrated human plasma. Usually no clotting takes place, and the fluids are ready to have their toxicity tested. Occasionally a weak coagulant activity may be noted. When this occurs, the fluids must be treated once with an equal volume of 1.4 normal hydrochloric acid to precipitate the clotting factor, which is then separated as above. The fluids after neutralization are now certain to be coagulant free.

#### TOXICITY OF THE FRACTIONS

Toxicity tests are made by subcutaneous injections into mice weighing from 15 to 20 Gm. In such animals the average minimal lethal dose of tiger snake venom acting within twenty-four hours is approximately 0.5 c.e. of 1:100,000 solution, or 0.005 mg. It was found that subcutaneous injections of the first supernatant solution, appropriately diluted, killed mice in amounts between 0.75 and 1.0 c.e. of 1:100,000 dilution, which is close to the lethal action of tiger snake venom. The small difference in degree of toxicity between the first supernatant solution and the whole venom solution is mostly accounted for in the toxicity of the second supernatant solution, which was lethal in amounts of 2 c.e. The third supernatant fluid has no significant amount of toxin when injected in doses as large as 3 c.e., the maximal amount of any fluid that can safely be injected at one time in a 15 to 20 Gm. mouse.

In testing the toxicity of the thrice-precipitated fractions (which contain the clotting factor) various preparations were made from amounts of whole venom up to 15 mg., which is equivalent to 3,000 minimal lethal doses in mice. Subcutaneously, inoculated as neutralized solutions in volumes of 2 to 3 c.e., these preparations were found to be harmless. Such mice were observed for a period of one month without their showing any ill effects.

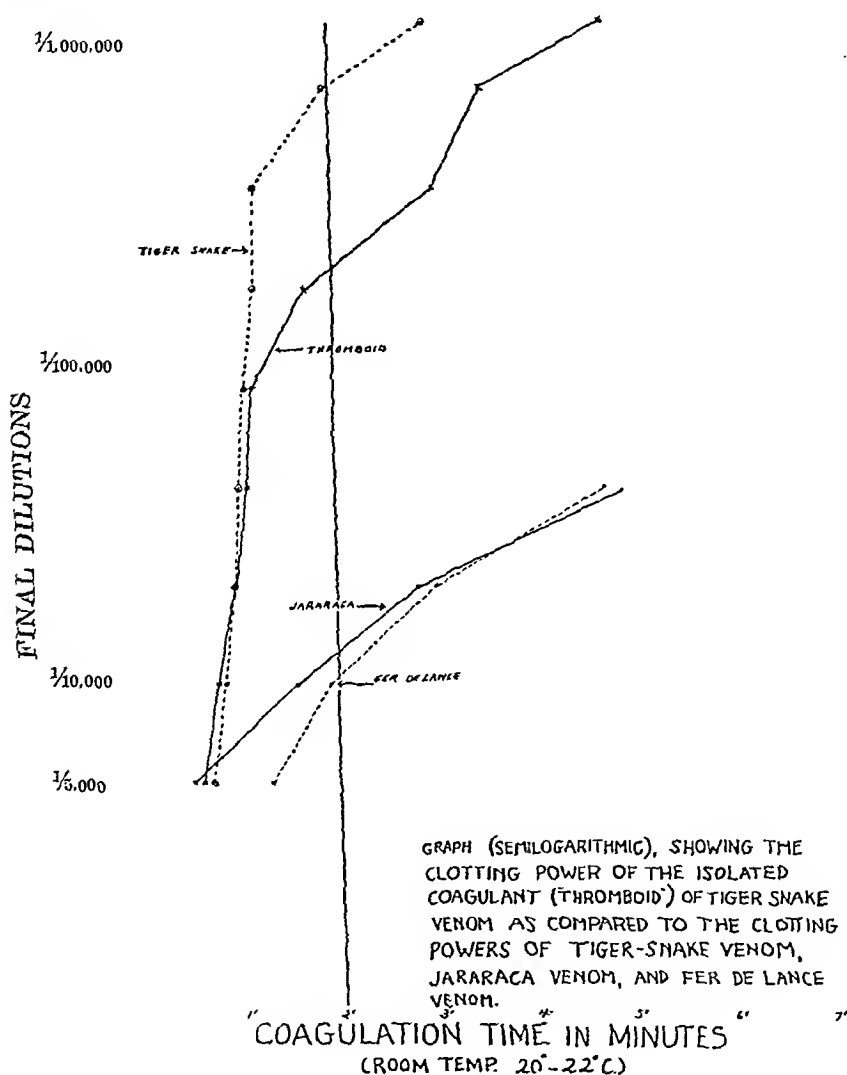


Fig. 1.

#### THE COAGULANT ACTION OF THE PRECIPITATED FRACTION\*

For testing the clotting power of the precipitated fraction, which contains the coagulant factor, a rack of small tubes was set up, each containing 0.1 c.e. of progressive dilutions of the fraction, each tube having one-half the strength of the preceding one. Considering the dilution of this fraction, when the volume

\*The term "thromboid" has been applied by us to the precipitated fraction of tiger snake venom solution because of its seemingly thrombinlike action upon citrated plasma without the addition of calcium.

is brought up to that of the original venom solution, to be 1:1,000, the resultant final titers after 0.4 c.c. of citrated human plasma has been added to each tube, ranged from 1:5,000 to 1:1,280,000. Control tubes were set up with similar dilutions of the tiger snake venom from which the fraction was made. It was noted that up to the titer of 1:100,000 the coagulant fraction was just as powerful as the parent substance (tiger snake venom), with clot formation taking place between thirty and ninety seconds, depending on the dilution. In higher dilutions, however, the coagulant fraction rapidly becomes weaker as compared to the whole venom; thus, at a dilution of 1:300,000 the clotting time is twice as long for the fraction as it is for the whole venom. It would, therefore, seem that there is some destructive action by action upon tiger snake venom in the separation of the coagulant factor. It must be borne in mind, however, that the latter was not dried and weighed, and it is reasonable to assume a more powerful action for the coagulant fraction if this were done.

Clotting tests were also made with a purified fibrinogen solution, prepared by triple precipitation with sodium chloride of Berkefeld-filtered citrated plasma.<sup>6</sup> When tiger snake venom or its coagulant fraction is added to the fibrinogen, no coagulation takes place, indicating that these reagents do not act like thrombin. But the addition of prothrombin, prepared by the action of carbon dioxide upon Berkefeld-filtered citrated plasma,<sup>9</sup> enables clotting to take place readily. Hence it appears that tiger snake venom and its coagulant fraction act like thrombokinase, rapidly activating prothrombin into thrombin, without the need of calcium. However, the addition of calcium produces a greater acceleration of the coagulation time. This conception of the thrombokinase-like activity of tiger snake venom and of the coagulant factor derived from it is in agreement with the previous findings of Mellanby<sup>5</sup> and Eagle.<sup>4</sup>

It is interesting to compare the clotting action of tiger snake venom and its derivative coagulant with the potent coagulant venoms of *Bothrops atrox* (fer-de-lance) and *Bothrops jararaca*.<sup>\*</sup> Up to a 1:5,000 titer, jararaca venom has a greater clotting activity than tiger snake venom or its derivative, but then it quickly loses its superiority and at a dilution of 1:10,000 and beyond it is distinctly weaker. The action of fer-de-lance venom closely parallels that of jararaca venom (see Fig. 1). The coagulant activity of still another venom—that of the Russell viper—appears to be far inferior to any of the above-mentioned venoms.

When jararaca or fer-de-lance venom is added to a pure fibrinogen solution, rapid coagulation takes place, indicating that these venoms, unlike tiger snake venom or its derivative, exhibit a thrombin activity.

In comparing the nature of the clots formed by the various venom coagulants, it was found that those produced by tiger snake venom and its derivative are firm and difficult to dislodge, whereas the venoms of the *Bothrops* group produce softer clots, which easily slide down the side of the tube when the latter is inverted. Furthermore, the clots of the *Bothrops* group undergo lysis within twelve hours, while those formed by the tiger snake venom and its derivative do not become fluid until after forty-eight hours.

<sup>\*</sup>For the separation of the coagulant from the toxic factor of the *Bothrops* group of venoms, the method described in this paper is not suitable. For the jararaca venom a method has already been devised.<sup>10</sup>



## SUMMARY

A method has been described for completely separating the coagulant from the toxic factor of the venom of the Australian tiger snake (*Notechis scutatus*). Neither of the fractions was prepared in a dry state, nor was any attempt made to determine its chemistry.

The coagulant fraction is approximately equal in potency to its parent substance up to dilutions of 1:100,000, but in still higher dilutions there is a deterioration in the potency of the fraction. Injected subcutaneously into mice, the coagulant factor prepared from as much as 15 mg. of tiger snake venom, which is equivalent to 3,000 minimal lethal doses, is harmless.

The toxic fraction of the venom not only has no significant coagulant activity but also retains almost quantitatively the toxicity of the original venom.

In its action both tiger snake venom and its coagulant fraction behave like thrombokinase plus calcium, readily converting prothrombin to thrombin. They, therefore, readily clot citrated plasma. Unlike the *Bothrops* venoms, which have a thrombin action, they do not clot purified fibrinogen solutions; however, they will do so when prothrombin is added to the fibrinogen.

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## STUDIES ON THE DETECTION OF ABSCESSES AND TUMORS\*

### III. CONCENTRATION AND DETECTION OF A RADIOACTIVE SUBSTANCE IN ABSCESSES

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IN AN EARLIER paper a new method for the detection of abscesses or tumors was described. It consisted of the injection of substances which localize in or around abscess or tumor tissue.<sup>1</sup> These substances were made artificially radioactive and their localization was detected by a sensitive electron counter, which registered the points of highest concentration of gamma rays in the body. The blue dye T 1824 was employed. Its concentration in abscessed and normal tissue and various organs was described, and a theory on the mechanism of the localization process, based on physicochemical interpretations, was proposed.<sup>2</sup> Since the purpose of this work was to prepare a radioactive substance, which would localize preferentially in abscess and tumor tissue, and thus be available for diagnostic purposes in man, our attention was turned to other dye derivatives in order to find a more suitable carrier for the radioactive atom.

The various dyestuffs which have been used for studying localization phenomena are mainly vis-azo acid dyes containing three to five sulfonic acid groups. In various studies made on these compounds in order to correlate chemical structure and physiologic activity, the number of sulfonic acid groups in the molecule plays an important role.<sup>3, 4</sup> The most readily available sulfonic acid derivative related to the bis-azo dyes is the disodium 1-amino 8-hydroxy 3,6-disulfonate (H acid), which is used as an intermediate in the preparation of trypan blue and other acid azo dyes. H acid has been found to have an affinity for serum proteins<sup>5</sup> and also to localize in mouse carcinomas.<sup>6</sup>

Because of the ease of bromination, H acid was used in the investigation described in the present paper. It was brominated in aqueous solution, with inactive or with radioactive bromine, and these solutions, after the proper preparation, were injected intravenously into dogs having an abscessed thigh. The localization of the brominated H acid was determined by chemical analysis, while the concentration process of the radioactive compound was followed in the live animal by a Geiger-Mueller counter according to a method described in a previous paper.<sup>1</sup>

*Experimental.*—Disodium 1-amino 8-hydroxy naphthalene 3,6-disulfonate was obtained from Eastman Kodak Co. The sodium salt was recrystallized from water, and a 1 per cent solution was used. The bromination of the H acid,<sup>†</sup> injection into the experimental animals, and chemical analysis of the tissues were

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†The bromination of H acid in aqueous solution is accompanied by an oxidation to a 1,1-naphthoquinone derivative.

carried out by methods already described,<sup>2</sup> except that in the present experiments the calculated amount of silver acetate was added to the solution, which was heated to boiling to coagulate the silver bromide, and the solution was centrifuged and filtered to remove the precipitate. All free bromide ions were removed by this procedure.

The radioactive bromine, a mixture of the several possible isotopes, was generated from active lithium bromide by oxidation with potassium bromate. The lithium bromide was activated by exposure to a deuteron beam by means of the cyclotron.

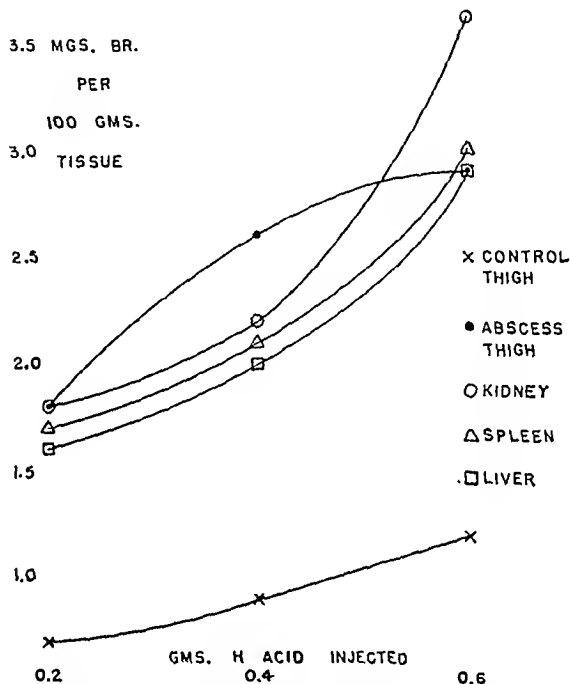


Chart 1.—Distribution of monobrominated H acid in the bodies of dogs.

The calculated amount of lithium bromide and potassium bromate required to monobrominate the H acid was dissolved in 10 to 15 ml. of water and placed in a modified distilling flask connected with a condenser. Three milliliters of 6 N sulfuric acid were added, and the reaction mixture was warmed gently. The bromine was distilled quantitatively into the flask containing the H acid. The bromination proceeded instantaneously, and the reaction was complete when the bromide-bromate solution became colorless, indicating that the lithium bromide had been completely oxidized. The brominated solutions thus prepared were treated in the same manner as the inactive preparations. Extreme care must be exercised to avoid contamination which would produce an increased count in the Geiger-Mueller apparatus.

The localization of the radioactive compound in the experimental animals was determined by the following procedures: An artificial abscess was produced in the thigh as described previously,<sup>1</sup> and twenty-four hours later the animals received an intravenous injection of the preparation; twenty-four hours later they were anesthetized by nembutal, and the following sites of the dog

were focused on the Geiger-Mueller tube: abscessed thigh, normal thigh, lower chest, upper chest, and head. The average total number of counts in a series of three-minute interval readings was used as a measure of the radioactive material concentrated in a given region. The Geiger-Mueller tube is so designed and shielded that the radiations which it may detect must come from the limited area studied. The radiations detected from the radioactive regions of the animal were primarily gamma rays.

TABLE I  
CHEMICAL ANALYSIS OF TISSUES AND ORGANS\*

| SAMPLE        | MG. OF Br PER 100 GM. OF WET TISSUE      |      |      |
|---------------|--|------|------|
|               | GRAMS OF MONOBROMINATED II ACID INJECTED |      |      |
|               | 0.2                                      | 0.4  | 0.6  |
| Abscess thigh | 1.80                                     | 2.60 | 2.90 |
| Normal thigh  | 0.70                                     | 0.90 | 1.20 |
| Kidney        | 1.80                                     | 2.10 | 3.60 |
| Spleen        | 1.70                                     | 2.20 | 3.00 |
| Liver         | 1.60                                     | 2.00 | 2.90 |

\*Animals sacrificed twenty-four hours after injection. The data listed here are the averages obtained from four animals for each concentration studied.

*Data.*—Table I tabulates the results of the chemical analysis of the tissues and various organs when 0.2, 0.4, and 0.6 Gm. of monobrominated II acid were injected. These data are illustrated graphically in Chart 1. The optimum concentration of monobrominated II acid on the basis of these results is the 0.4 Gm. level. With this dose, the concentration in the abscess was slightly higher or equal to the concentration found in spleen or kidney. At the 0.6 Gm. level the amount of bromine found in the spleen and kidney was greater than that found in the abscess. On the basis of the above results, 0.4 Gm. of II acid was used in the study made with the radioactive compound. The results of the localization of the radioactive II acid are given in Table II.

TABLE II  
DISTRIBUTION OF RADIOACTIVE MONOBROMINATED II ACID IN FOUR ANIMALS FOLLOWING INTRAVENOUS INJECTION OF 0.4 GM.

| SITE OF COUNT | AVERAGE COUNT PER THREE-MINUTE INTERVAL |     |               |     |
|---------------|---|-----|---------------|-----|
|               | PREPARATION A                           |     | PREPARATION B |     |
|               |   |     |               |     |
| Control*      | 16                                      | 19  | 22            | 19  |
| Abscess       | 57                                      | 125 | 106           | 105 |
| Normal thigh  | 31                                      | 54  | 53            | 51  |
| Lower chest   | 68                                      | 96  | 133           | 121 |
| Upper chest   | 65                                      | 109 | 105           | 90  |
| Head          | 54                                      | 112 | 85            | 64  |

\*Background count due to cosmic and other radiation.

The conclusion drawn from this series of experiments corroborates the chemical analysis. The region of the lower chest, occupied by the kidney and spleen, has a slightly higher concentration of radioactive material than the abscessed thigh in 3 of 4 experiments.

#### DISCUSSION

The experiments described, although still in the experimental stage, illustrate a new application of the artificially radioactive elements namely, as diag-

nostic indicators for regions of infection. There are many instances where a knowledge of the location of a focus of infection would be of the utmost importance to the surgeon. The preliminary studies made on the location of the brominated H acid in inflamed tissue partly realize this aim, although more experimental work must be done before the use of these indicators may be applied clinically.

The problems to be solved before clinical investigations become possible are (1) the preparation of suitable carriers for the radioactivation; (2) an extensive study of the localization of these carriers in various types of inflammation, tumor growths, and tissue abnormalities; and (3) the technical improvement of the Geiger-Mueller counter so that it may be easily handled and focused on limited areas. It is expected that once the various phases of research listed above are carried to a successful culmination, the radioactive isotope and the Geiger-Mueller counter will prove to be a valuable tool in the hands of the diagnostician.

#### SUMMARY

The bromination of disodium 1-amino 8 hydroxy naphthalene 3, 6-disulfonate with normal and radioactive bromine is described.

The localization of the inactive brominated derivative in abscess and normal tissue, spleen, kidney and liver, after intravenous injection of varying concentrations, was determined by chemical analysis. The concentration of the radioactive material was determined by locating the regions of highest radioactivity in the live animal by a Geiger-Mueller counter. The application of radioactive isotopes in the diagnosis of tissue abnormalities is discussed.

We want to express our gratitude to Doctors Simon and Slotin from the Department of Chemistry, the University of Chicago, for preparation of the radioactive lithium bromide.

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## STUDIES IN ABSORPTION OF UNDIGESTED PROTEIN\*

### IX. ABSORPTION FROM THE STOMACH AND ESOPHAGUS

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THE absorption of traces of unaltered protein occurs physiologically in fasting adults and children within a relatively short period after oral administration of the protein meal.<sup>1</sup> In studies by Brunnner and Walzer<sup>2</sup> fish protein appeared within fifteen minutes after its ingestion in the circulation of 93.8 per cent of the patients tested. In one instance, fish protein was detected in the blood stream two minutes after the consumption of the fish meal. The rapid absorption of other proteins, such as egg,<sup>3</sup> peanut,<sup>4</sup> and cottonseed<sup>5</sup> in an unaltered state, has also been demonstrated. Such absorption occurs regularly, not only following the ingestion of these substances but also following their administration by tube into the duodenum,<sup>6</sup> into the ileum and colon,<sup>7</sup> and into the rectum.<sup>8</sup>

Whether absorption of unaltered protein can occur from the stomach has long been a moot question.<sup>9</sup> In a study of 3 patients with gastric carcinoma with incomplete pyloric obstruction, Gray and Walzer<sup>4</sup> found the absorption rates to be considerably retarded in every instance. This retardation was attributed to mechanical interference with the passage of the ingested protein into the duodenum. Because of the pathologic changes of the gastric mucous membrane in these cases, no conclusions applicable to the normal stomach could be drawn from the investigations.

Further study of the question of gastric absorption was sought by comparing the absorption rates of peanut protein following oral and intraduodenal administration in a series of 14 selected patients.<sup>9</sup> Averages of 24.3 minutes and 18.6 minutes, respectively, were obtained following the oral and intraduodenal administrations of the protein meal. If it could be assumed that no absorption occurred from the stomach or esophagus, the average delay of five or six minutes associated with oral administration might be accounted for as the time needed for the passage of protein from the stomach into the duodenum in amounts sufficient to be absorbed from the small bowel. Previous experimental studies, however, did not eliminate the possibility that absorption of protein might be taking place from the stomach as well as from the small intestine. The need for further information on the subject of absorption from the stomach and esophagus, therefore, became apparent.

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As a result of the pioneer work of Straus it was found that the immunologic technique which had been previously employed to study the absorption of unaltered protein in human beings could be applied effectively to similar studies in the rhesus monkey.<sup>10</sup> This opened up new possibilities for experimental investigation of problems in absorption which had not been feasible in the human being. The present studies were, therefore, undertaken to investigate the question of absorption of unaltered protein from the stomach and esophagus in the rhesus monkey.

#### TECHNIQUE

The technique employed in these experiments was that described by Straus and Walzer in a previous communication.<sup>10</sup> A cutaneous site on a rhesus monkey was sensitized by the intracutaneous injection of a human serum containing skin-sensitizing antibodies (atopic reagins) for the antigen to be studied. After an interval of from twenty-four to forty-eight hours to permit fixation of the antibodies at the cutaneous site, the specifically related antigen was administered orally. The lighting-up of the sensitized cutaneous site with an urticarial reaction within a few minutes marked the entrance of unaltered protein into the circulation.

The *sensitizing serum* in these experiments was obtained from an asthmatic patient, "P," who manifested marked positive reactions to intracutaneous tests with dilute cottonseed extract. This serum showed an unusually high titer in atopic reagin content for cottonseed protein (1:1,024 by the dilution method of Coca and Grove).<sup>11</sup> To effect local cutaneous sensitization about 0.05 ml. of the undiluted human serum was injected intracutaneously on the thigh or chest of the monkey. An interval of one or two days was then allowed to elapse before the absorption experiments were performed. Food was withheld from the animals for at least twenty-four hours previous to the experiment, but small amounts of water were given. The *protein test meal* consisted of a cottonseed "milk" which was made by mixing 10 Gm. of raw ground cottonseed in 30 ml. of tap water. The method of administration of the test meal and the amount employed varied with the experiment. The *absorption time* was the interval which elapsed between the administration of the test meal and the onset of the reaction at the sensitized cutaneous site.

#### ABSORPTION OF COTTONSEED PROTEIN FROM THE STOMACH

*Experiment I.*—This experiment was designed to determine whether or not absorption can occur from the stomach in the rhesus monkey. The animal employed in this study was a female monkey, weighing 4 kg. The details of the experiment are presented in their chronologic order as follows:

November 5, 1937. 9:00 A.M. A cutaneous site on the left chest of the monkey was passively sensitized to cottonseed antigen with an intracutaneous injection of 0.05 ml. of "P" serum.

November 6, 1937. 9:30 A.M. Two grains of veterinary nembutal were administered intravenously. Immediate narcosis resulted.

10:00 A.M. The abdomen was opened. Two clamps were placed at the pyloric end of the stomach. The stomach was completely sectioned between these clamps. The clamps were left in situ.

10:07 A.M. A rubber catheter was introduced into the stomach through the nose. Thirty milliliters of cottonseed "milk" was then injected, through the tube, into the stomach.

10:18 A.M. Wheal formation started to develop at the sensitized cutaneous site, indicating the absorption of the cottonseed protein into the circulation.

The *absorption time* of the cottonseed protein from the stomach in this animal was eleven minutes.

*Experiment II.*—Before definitely concluding that the positive result obtained in the foregoing experiment was based on absorption of the antigen from the stomach, it was necessary to exclude the esophagus as a possible site of absorption. In this experiment, therefore, the stomach was "isolated" from the esophagus as well as from the duodenum. A male monkey, weighing 3.5 kg., was used in this study.

November 27, 1937. 9:00 A.M. A cutaneous site on the left thigh of the monkey was sensitized to cottonseed with "P" serum.

November 28, 1937. 9:30 A.M. Narcosis was induced by the intravenous injection of 2 grains of nembutal.

9:35 A.M. The abdomen was opened. Two clamps were applied just below the cardio-esophageal orifice, and the stomach was completely sectioned between these two clamps. Just proximal to the pyloroduodenal junction the stomach was completely sectioned between two clamps applied at this site. All clamps were left in situ.

9:56 A.M. Fourteen milliliters of cottonseed "milk" were injected by syringe directly into the lumen of the stomach, through a fine needle penetrating the gastric wall. The stomach was only partially filled by this test meal.

9:58 A.M. Wheal formation started to develop at the sensitized cutaneous site, marking the entrance of cottonseed protein into the circulation.

The *absorption time* of the cottonseed protein from the stomach in this animal was two minutes.

#### ABSORPTION OF COTTONSEED PROTEIN FROM THE ESOPHAGUS

*Experiment III.*—This experiment was designed to determine whether or not absorption can occur from the esophagus in the rhesus monkey. For this experiment a male animal, weighing 4.2 kg., was used.

December 3, 1937. 9:00 A.M. A cutaneous site on the right thigh of the animal was passively sensitized to cottonseed with "P" serum.

December 5, 1937. 9:20 A.M. Narcosis was induced with 2 grains of nembutal, injected intravenously.

9:30 A.M. The upper and the lower ends of the esophagus were exposed. The lower end was sectioned between two clamps placed proximal to the cardio-esophageal junction. The upper end was similarly sectioned between two clamps placed approximately one-half inch below the pharyngo-esophageal junction. The clamps were left in situ.

9:56 A.M. The clamp at the upper end of the esophagus was momentarily released, and 7 ml. of cottonseed "milk" were introduced into the esophagus through its exposed upper opening. The clamp was then replaced in its original position.



10:06 A.M. A positive reaction started to develop at the sensitized cutaneous site, indicating the entrance of the cottonseed protein into the circulation.

The absorption time of the cottonseed protein from the esophagus in this animal was ten minutes.

#### DISCUSSION

The experiments were performed on the monkey because these studies obviously could not be done on man. In previous investigations,<sup>10</sup> however, it has been repeatedly demonstrated that the phenomenon of absorption of unaltered protein in the monkey closely simulates that observed in human beings. Moreover, the experimental studies<sup>7, 12</sup> on mucous membrane hypersensitiveness after passive local sensitization have also shown remarkable similarities in both man and the monkey. Under the circumstances, it is reasonable to assume that the absorption of unaltered protein from the "isolated" stomach and "isolated" esophagus observed in the monkey could be demonstrated in human beings under similar experimental conditions.

Attention has been called to the findings revealed in a previous study on human beings that the average absorption time following oral administration of antigen is longer by five or six minutes than the intraduodenal rate.<sup>6</sup> In the light of the present experiments this difference in absorption rate, which might be assumed to be the time needed for the protein to pass from the esophagus and stomach into the duodenum, is now subject to another interpretation. The possibility must be entertained that under certain circumstances the absorption time following oral administration of the antigen may represent the rate of absorption of unaltered antigen from the stomach and even from the esophagus. At any rate, this route of entry of unaltered antigen into the circulation cannot be excluded as a possibility when the antigen is taken orally. In fact, the experimental evidence now at hand indicates that antigens may be absorbed from any part of the alimentary tract, though the rate of absorption may vary somewhat in the different organs.

The amount of antigen necessary to produce the allergic reaction has been shown to be infinitesimally small. In man, Brunner and Baron, in unpublished experiments, have demonstrated that the intravenous injection of cottonseed extract containing 0.0001 mg. of nitrogen is sufficient to induce a reaction at a cutaneous site previously sensitized with the serum used in the present experiments. Using the same serum in experiments on monkeys we, in unpublished experiments, have found that the intravenous injection of cottonseed extract containing 0.0002 mg. of nitrogen was usually sufficient to elicit a reaction at a sensitized cutaneous site. It is obvious, therefore, that only minute amounts of protein need be absorbed into the circulation to produce a reaction at a passively sensitized site. From the point of view of clinical allergy, however, these traces of absorbed unaltered protein may be sufficient to produce severe and even fatal reactions in sensitive patients.

#### CONCLUSION

The absorption of unaltered protein has been experimentally demonstrated to occur from the esophagus and from the fasting stomach in the rhesus monkey.

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## THE IDENTIFICATION OF *BLASTOMYCOIDES HISTOLYTICA* IN THREE INFECTIONS OF THE CENTRAL NERVOUS SYSTEM\*

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Synonyms: *Torula histolytica* (Stoddard and Cutler, 1916), *Cryptococcus histolyticus* (Freeman and Weidman, 1923), *Torulopsis histolytica* (Castellani and Jacono, 1933), *Debaryomyces hominis* (Vuillemin-Todd and Herrmann, 1936).

THE presence of *Blastomycoides histolytica*<sup>1</sup> (Stoddard and Cutler, 1916), or a closely allied species, has been reported according to Magruder<sup>2</sup> as the causative agent in 66 cases involving the central nervous system, of which three were found at the University of Virginia Hospital. Recently, Gray<sup>3</sup> added two more cases which occurred in South Africa, and Goldberg added an-

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One other probable unreported case, at the Medical College of Virginia. A histopathologic diagnosis of Fusch, but was not confirmed. These cases will be reviewed from the neurologic standpoint by Dr. James Asa Shield in a separate paper.

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other one in New York. I wish to report here the bacteriologic and pathologic data on three other cases, bringing the total number\* of recorded cases in the literature to 72, and that in Virginia to six.

Since the chaos in the nomenclature of the yeastlike organisms causing these infections has been deplored by Shaw† and others, the synonyms listed above are given. Shaw has pointed out also that not as many species exist as are now recorded and stressed the importance of recognizing their protean manifestations. Further, a review of the literature revealed the fact that seldom has there been a complete bacteriologic description of this yeast accompanying the histologic and clinical studies on the same case, although very adequate cultural or tissue descriptions on separate cases have been made.

CASE 1.—In this case studied by us, *Blastomycoides histolytica* was demonstrated in post-mortem sections and cultures, and was confirmed by mouse experiments as suggested by Crone and associates,‡ in order to standardize a routine procedure for diagnostic purposes. No ante-mortem bacteriologic work was done, although a routine urinalysis showed the presence of yeastlike cells, which were not identified. The clinical diagnosis was that of a brain tumor.

Pathologic Studies:‡ A post-mortem examination was permitted on the head only. No gross pathologic findings suggested torulosis. Microscopic examination of the piaarachnoid tissue showed a marked diffuse cellular infiltration. These infiltrating cells were small round cells and epithelioid-like cells with finely granular eosinophilic cytoplasm and eccentrically placed vesicular lobulated nuclei. There tended to be paravascular granulation tissue nodules composed of the above cell types, both in the meninges and about the vessels within the brain tissue proper. The epithelioid-like cells formed an inner zone about the vessel, while the round cells formed an outer zone. Multinucleated giant cells with eosinophilic cytoplasm containing round clear bodies were found in these nodules. About the vessels in the basal ganglia were "cystlike" areas made of numerous round bodies with doubly refractile borders and a central ground glass substance when stained with hematoxylin and eosin. With Gram's stain these bodies took a deep blue color and were identified as yeastlike cells, probably torula. There was no cellular reaction in these areas. These bodies were also found in the subarachnoid tissue where about them was a marked cellular reaction, focal areas of diffuse hemorrhage, and phagocytosis of some by the above-described multinucleated giant cells (Fig. 1.).

Bacteriologic Studies: A routine culture on blood agar plate of a meningeal swab taken at autopsy revealed, after forty-eight hours, a mucoid, colorless growth. Microscopically, the presence of yeastlike organisms was noted with many budding forms and well-defined cell walls. Transplants on Sabouraud's media and corn meal agar plates showed sufficient growth at room temperature within forty-eight hours for preliminary studies. The growth on corn meal agar always appeared white. On Sabouraud's agar early growth was cream-colored, later becoming yellow, then brown. A mucoid consistency of early colonies was gradually lost. No fermentation of dextrose, lactose, saccharose, maltose, mannite, inosite, arabinose, or xylose occurred. Gelatin was not liquefied. No differences were seen in cultures grown under reduced oxygen tension from those not submitted to this atmosphere. Microscopically, gram-positive, round, and oval budding forms, 4 to 6 microns in diameter, with thick cell walls were observed. No ascospores, capsules, or mycelium were demonstrated (Fig. 2).

Animal Experiments: Six mice were injected intraperitoneally with 0.5 c.c. of saline suspension of a seventy-two-hour culture on Sabouraud's agar at room temperature. The density of the injection suspension was equivalent to standard No. 3 on MacFarland's

\*Since the acceptance of this paper for publication, six additional cases<sup>10</sup> of torula infection of the central nervous system have been reported.

†Personal communication from Dr. Frederick W. Shaw.

‡Published with the approval of Dr. Frank L. Apperly, Department of Pathology, Medical College of Virginia, Richmond.

nephelometer. Seven days later an apparently normal mouse was killed. Yeastlike organisms were found in the viscera, lung, heart, brain, and cord as proved by direct smears, cultures, and histologic sections. Grossly and microscopically, no marked changes of the tissues were noted. On direct smears pleomorphism of the organisms (2 to 12  $\mu$  diameter) and capsule formations (0.5 to 1.0  $\mu$  thickness) were observed, but no ascospores or mycelium. *Blastomycoides histolytica* was recovered in the cultures of the vital organs listed above, with

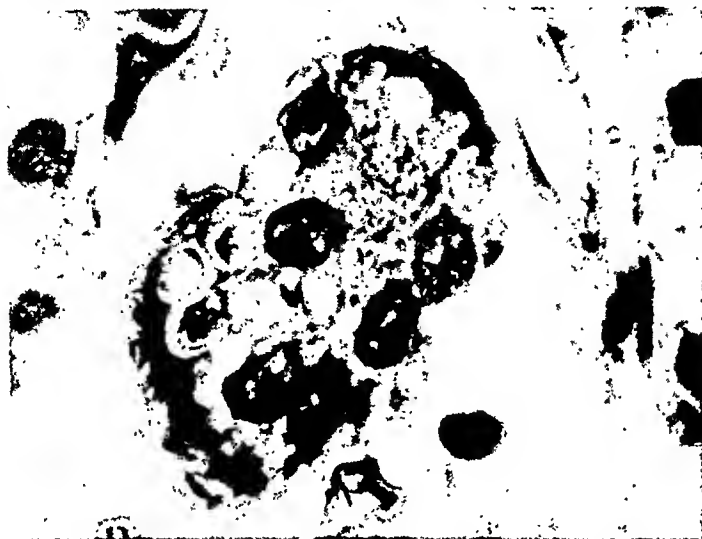


Fig. 1.—Section of human brain. Organisms within giant cell. Hematoxylin and eosin stain ( $\times 2,200$ ).

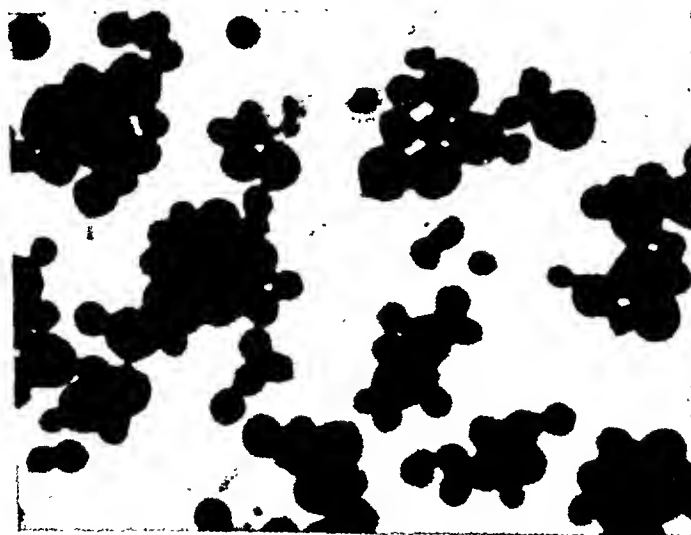


Fig. 2.—Smear preparation of forty-eight-hour culture on Sabouraud's agar from human meninges. Gram's stain ( $\times 2,200$ ).

similar growth characteristics of the primary cultures. Four of the remaining inoculated mice died within the following three weeks. The gross and microscopic pathology of yeast meningitis, with a generalized infection characterized by gelatinous tumors with slight inflammatory reactions, marked endothelial hyperplasia, foreign body giant cells, "cystlike" areas, and occasional caseation was seen. *Blastomycoides histolytica* was recovered again by

cultural means. The sixth mouse was killed after four months, although it appeared normal. No gross or microscopic pathology was noted, and no organisms were seen on direct smears. *Blastomycoides histolytica* was recovered, however, in cultures of the lung and liver. Neither agglutinins nor precipitins were demonstrated in the sera of rabbits immunized against antigens prepared as were those of Benham,<sup>5</sup> and Lamb and Lamb<sup>6</sup> (Figs. 3 and 4).



Fig. 3.—Section of mouse brain showing organisms in meningeal exudate. Hematoxylin and eosin stain ( $\times 2,200$ ).

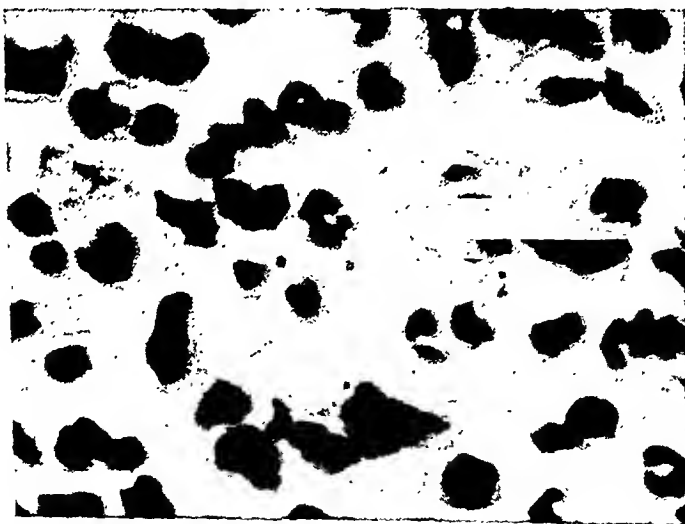


Fig. 4.—Section of mouse lung. Hematoxylin and eosin stain ( $\times 2,200$ ).

CASE 2.—This case was diagnosed as meningitis due to yeast infection on March 23, 1938. The patient received specific treatment with iodides and was dismissed. Since then she has remained in apparent good health, except for the blindness which developed during the illness.

Bacteriologic Studies: The first isolation of yeastlike organisms was made from the spinal fluid by cultural means similar to those already given. No animal experiments were run at this time.

Recently upon readmission of this patient to the hospital for observation, the presence of the same yeast was shown in urine and spinal fluid as proved by mouse inoculations with pathologic studies and cultures to be *Blastomycoides histolytica*. Results of the blood, sputum, and stool cultures were negative. No agglutinins or precipitins were demonstrated in the patient's sera.

CASE 3.—Until *Blastomycoides histolytica* was isolated from two specimens of spinal fluid just prior to death, clinically this case was thought to resemble more closely tuberculous meningitis, although no acid-fast bacilli were found on smear preparations or in cultures. Only ante-mortem bacteriologic studies were made, since an autopsy was not granted.

Bacteriologic Studies: The identification of *Blastomycoides histolytica* in two specimens of spinal fluids was accomplished by the same procedures as previously given. In direct microscopic studies no familiar budding forms were seen, although ill-defined lymphocyte-like cells with thick refractile walls were noted in moist preparations and smears stained by Gram's method. The demonstration of acid-fast bacilli was accomplished neither by microscopic examinations nor by cultures on Miraglia's and Petragani's media. Growth of *Blastomycoides histolytica* was inhibited on Petragani's media containing malachite green, although it was profuse on Miraglia's at 37° C. Fungous and acid-fast cultures on the urine collected just before death proved negative.

#### SUMMARY

The increasing number of reports of yeast infections of the central nervous system, especially in Virginia, lead us to believe the condition not to be an uncommon one. Since uncertain clinical diagnoses have been so frequently and generally made upon these cases, emphasis should be placed upon bacteriologic studies prior to death and after it. Many, but not all, stages in the development of *Blastomycoides histolytica*, as pointed out by Todd and Herrmann,<sup>7</sup> were seen. We feel, as do Longmire and Goodwin,<sup>8</sup> that the character of the lesions produced by this yeast is the result of an interrelation of factors, such as the virulence of the strain of organisms, the resistance of the host, and the hypersensitivity of the host. Further studies in cytology and on certain immunologic properties of this organism have been undertaken.

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# CLINICAL CHEMISTRY

## CHEMICAL STUDIES IN DELIRIUM TREMENS\*

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TREATMENT of delirium tremens<sup>1, 2</sup> has consisted mainly of procedures intended to dehydrate the patient. The post-mortem finding of cerebral edema in a large percentage of cases provided the basis for this type of therapy. This regimen has persisted in spite of the fact that many patients are obviously clinically dehydrated.

Attention was called to the unphysiologic nature of dehydration therapy in delirium tremens by Bowman and his co-workers,<sup>3</sup> who showed that dehydrated excited alcoholic persons were benefited by oral or parenteral administration of hypertonic sodium chloride. In a later article,<sup>4</sup> Bowman, Wortis, and Keiser advocated the use of large amounts of fluids, in addition to carbohydrates and sodium chloride, in an effort to readjust metabolism and blood chemistry. On the basis of unpublished data, they stated that the blood chloride and sodium were lowered and lactic acid was increased. The present study was undertaken in an effort to evaluate the physiologic and chemical basis for their recommendations.

### MATERIAL AND METHODS

The criteria for the diagnosis of delirium tremens were: (1) History of alcoholism. (2) Visual and auditory hallucinations. (3) Delirium. (4) Excitement. (5) Disorientation. (6) Tremors of the hands and tongue. Patients admitted to the hospital with these findings were studied immediately on admission and after recovery, the latter study serving as control. Treatment varied but in general consisted of sodium chloride, fluids, thiamine, and nicotinic acid administered orally or parenterally. All patients in this series were males.

Blood sugar was determined by the method of Folin-Wu<sup>5</sup>; urea nitrogen by Karr's method<sup>6</sup>; serum proteins colorimetrically by means of the biuret reaction method of Kingsley<sup>7</sup>; and hemoglobin by means of the photoelectric photometer, according to Sheard, Sanford, and Osterberg.<sup>8</sup> Serum chloride was measured by the method of Van Slyke, as modified by Wilson and Ball,<sup>9</sup> total base by the electro dialysis method of Keys,<sup>10</sup> and carbon dioxide combining power by the method of Van Slyke.<sup>11</sup> Spinal fluid proteins were measured by Looney and Walsh's turbidimetric method.<sup>12</sup> Available fluid was determined by the method of Crandall and Anderson<sup>13</sup> utilizing sodium thiocyanate. Hematoerit values were obtained by use of the method described by Peters and Van Slyke.<sup>11</sup>

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TABLE I  
DETAILED CHEMISTRY OF PATIENTS STUDIED  
"A" and "R" below refer to values on admission and after recovery, respectively.

| PA-<br>TIENT |  | DATE     | BLOOD CHEMISTRY     |                                     |                 |      |                            |                             |     |                                    |     |   | SPINAL FLUID CHEMISTRY  |     |                          |                        |                     |                            |                            |
|--------------|--|----------|---------------------|-------------------------------------|-----------------|------|----------------------------|-----------------------------|-----|------------------------------------|-----|---|-------------------------|-----|--------------------------|------------------------|---------------------|----------------------------|----------------------------|
|              |  |          | SUGAR<br>(MG.<br>%) | UREA<br>NITRO-<br>GEN<br>(MG.<br>%) | HEMATO-<br>CRIT |      | HEMO-<br>GLOBIN<br>(GM. %) | TOTAL<br>PROTEIN<br>(GM. %) |     | AVAILABLE<br>FLUID<br>(1m./SQ. M.) |     | CO <sub>2</sub><br>COMBIN-<br>ING<br>POWER<br>(VOL.<br>%) | CHLORIDES<br>(MEQ./1m.) |     | TOTAL BASE<br>(MEQ./1m.) | CHLORIDES<br>(MEQ./L.) | SUGAR<br>(MG.<br>%) | PRO-<br>TEIN<br>(MG.<br>%) | TOTAL<br>BASE<br>(MEQ./L.) |
|              |  |          |                     |                                     | A               | R    |                            | A                           | R   | A                                  | R   |   | A                       | R   |                          |                        |                     |                            |                            |
| 1            |  | 12/ 7/39 | 70                  | 11                                  | 60              | 16.0 | -                          | -                           | -   | -                                  | -   | 45  | 94.0                    | -   | 121                      | 55                     | -                   | -                          |                            |
| 2            |  | 12/11/39 | 104                 | 11                                  | -               | 16.8 | 6.0                        | -                           | -   | -                                  | -   | 48  | 97.4                    | -   | 122                      | 80                     | 68                  | -                          |                            |
| 3            |  | 12/13/39 | -                   | -                                   | -               | -    | -                          | -                           | -   | -                                  | -   | -   | -                       | -   | -                        | -                      | -                   | -                          |                            |
| 3            |  | 12/22/39 | 126                 | 19                                  | -               | 14.0 | 6.8                        | -                           | 8.0 | -                                  | -   | 48  | 86.9                    | -   | -                        | -                      | -                   | -                          |                            |
| 4            |  | 12/28/39 | -                   | -                                   | 39              | 12.7 | 5.5                        | 7.4                         | -   | -                                  | -   | 48  | 92.3                    | -   | 124                      | 60                     | 46                  | -                          |                            |
| 4            |  | 12/11/39 | 63                  | 9                                   | -               | -    | -                          | -                           | -   | -                                  | -   | -   | -                       | -   | -                        | -                      | -                   | -                          |                            |
| 5            |  | 12/18/39 | -                   | -                                   | -               | -    | -                          | -                           | -   | -                                  | -   | -   | -                       | -   | -                        | -                      | -                   | -                          |                            |
| 5            |  | 12/11/39 | 80                  | 7                                   | -               | 14.8 | 4.2                        | -                           | -   | -                                  | -   | 66  | 88.8                    | -   | 112                      | 70                     | 32                  | -                          |                            |
| 6            |  | 12/18/39 | -                   | -                                   | -               | 15.6 | 8.2                        | -                           | 7.4 | -                                  | -   | 44  | 85.0                    | 154 | 117                      | 127                    | 50                  | 164                        |                            |
| 6            |  | 12/29/39 | 212                 | 9                                   | 42              | -    | -                          | -                           | -   | -                                  | -   | -   | -                       | -   | -                        | -                      | -                   | -                          |                            |
| 7            |  | 1/ 2/40  | 123                 | -                                   | 37              | 14.4 | 7.4                        | 6.5                         | 6.7 | 9.9                                | -   | -   | 97.0                    | -   | 124                      | 82                     | 38                  | -                          |                            |
| 7            |  | 1/ 4/40  | 84                  | 7                                   | 43              | 14.4 | 7.4                        | 9.0                         | 8.9 | 8.8                                | 8.8 | 38  | 98.4                    | 161 | 124                      | 67                     | 34                  | -                          |                            |
| 8            |  | 1/10/40  | -                   | -                                   | 41              | 14.0 | 7.8                        | -                           | 6.6 | -                                  | -   | 41  | 82.8                    | 138 | 113                      | 89                     | 34                  | -                          |                            |
| 9            |  | 1/10/40  | 109                 | 11                                  | 43              | 14.8 | 8.9                        | 6.5                         | 8.9 | 8.8                                | 8.8 | 49  | 98.4                    | 161 | 124                      | 67                     | 34                  | -                          |                            |
| 10           |  | 1/18/40  | -                   | -                                   | 44              | 13.6 | -                          | -                           | -   | -                                  | -   | -   | -                       | -   | -                        | -                      | -                   | -                          |                            |
| 10           |  | 2/ 1/40  | 75                  | 7                                   | 42              | 14.1 | 6.4                        | -                           | -   | -                                  | -   | 33  | 97.0                    | 151 | 125                      | 75                     | 56                  | -                          |                            |
| 11           |  | 2/ 6/40  | 132                 | 25                                  | 42              | 10.5 | 7.6                        | -                           | 6.6 | 39                                 | 39  | 39  | 90.7                    | 155 | 119                      | 123                    | 56                  | -                          |                            |
| 12           |  | 7/27/40  | 96                  | 12                                  | 43              | 14.4 | 9.4                        | -                           | 7.8 | 40                                 | 40  | 40  | 98.4                    | 155 | 124                      | 77                     | 50                  | 165                        |                            |
| 13           |  | 8/ 1/40  | -                   | -                                   | 35              | 14.0 | -                          | 8.4                         | -   | 9.5                                | -   | -   | -                       | -   | -                        | -                      | -                   | -                          |                            |
| 13           |  | 8/ 1/40  | 121                 | 13                                  | 45              | 15.2 | 7.4                        | -                           | 8.7 | 9.1                                | 55  | 55  | 89.7                    | 159 | 119                      | 84                     | 53                  | 162                        |                            |
| 14           |  | 8/ 6/40  | -                   | -                                   | 42              | 14.4 | -                          | 7.1                         | -   | 9.1                                | -   | -   | -                       | -   | -                        | -                      | -                   | -                          |                            |
| 14           |  | 8/ 2/40  | 96                  | 9                                   | 46              | 15.4 | 7.7                        | 6.7                         | 6.9 | 9.1                                | 53  | 53  | 92.2                    | -   | 124                      | 72                     | 41                  | 159                        |                            |
| 15           |  | 8/ 6/40  | -                   | -                                   | 36              | 14.0 | -                          | 6.7                         | 8.9 | 9.1                                | 43  | 43  | 90.5                    | 148 | 127                      | 89                     | 31                  | -                          |                            |
| 15           |  | 7/28/40  | 100                 | 15                                  | 41              | 14.6 | 6.5                        | -                           | -   | -                                  | -   | -   | -                       | -   | -                        | -                      | -                   | -                          |                            |



## RESULTS

On admission serum chloride and carbon dioxide combining power were below normal in nearly all patients. Total base and available fluid determinations showed low or low normal values predominantly. Hemoglobin and serum protein concentrations, together with hematocrit values, were increased. The spinal fluid also showed changes. Chlorides were decreased and protein was increased. On recovery the concentrations of the substances studied returned to normal, with the occasional exception of the serum chloride. In four cases the recovery values for chloride were slightly below normal. The blood sugar and urea nitrogen showed no significant change.

All patients save one recovered; patient 8 died within twenty-four hours after admission as a result of lobar pneumonia. The pneumonia may have contributed to the factors causing the low serum chloride.

TABLE II

MEAN VALUES AND THEIR STANDARD ERRORS OF THE BLOOD CONSTITUENTS

In computing the value for protein, the values for patient 7 were omitted. "A" refers to admission values; "R" to values after recovery.

|       | HEMATO-CRIT |      | HEMO-GLOBIN (GM. %) |      | PROTEINS (GM. %) |      | AVAILABLE FLUID (L./M.) |      | CHLORIDES (MEQ./L.) |      | TOTAL BASE (MEQ./L.) |      | CO <sub>2</sub> COMBINING POWER |
|-------|-------------|------|---------------------|------|------------------|------|-------------------------|------|---------------------|------|----------------------|------|---------------------------------|
|       | A           | R    | A                   | R    | A                | R    | A                       | R    | A                   | R    | A                    | R    | A                               |
| Mean  | 45.2        | 39.1 | 14.5                | 13.3 | 8.1              | 7.1  | 7.7                     | 9.2  | 92.5                | 99.0 | 152                  | 163  | 46                              |
| S. E. | ±1.5        | ±1.8 | ±0.3                | ±0.5 | ±0.3             | ±0.3 | ±0.3                    | ±0.3 | ±1.3                | ±1.6 | ±2.1                 | ±2.4 |                                 |

## DISCUSSION

The ten patients on whom available fluid studies were performed on admission showed clinical evidence of dehydration. The available fluid values were at or below the accepted lower limits. In six of the ten, available fluid values were less than normal, and in the remainder low normal. After recovery the values were all within normal limits. Comparison of the mean values on admission and after recovery by the method described by Student using Fisher's<sup>14</sup> table of *t* showed a significant difference. Crandall and Anderson,<sup>13</sup> and Gregerson and Stewart,<sup>15</sup> designate as available fluid, rather than extracellular fluid, the body water determined by the thiocyanate method. Normal values for available fluid are said to lie within the range of 22 to 29 per cent of the body weight, or between 8.7 and 9.2 liters per square meter of body surface area. Crandall and Anderson claim that the estimate of available fluid based on surface area is more accurate and less subject to variation than that based on body weight. This method was used.

Hemoconcentration existed in most, if not all, of the patients on admission. Hemoglobin, protein, and hematocrit values were high on admission when compared with the results of similar studies after recovery. Statistical analysis of the mean values on admission and after recovery showed the differences to be significant.

Total base and chloride determinations revealed hypochloremia and some evidence of base deficiency on admission. In only two of the fifteen patients

studied did chloride fall within normal limits. These two showed figures at the lower limits of normal of 98.0 meq. per liter. In four patients values between 96.0 and 98.0 meq. per liter were observed. All others showed chloride values below 94.0 meq. per liter. Comparison of the mean values of chloride on admission and after recovery indicated the differences to be highly significant. Total base on admission showed a marked departure from normal in only two patients. Recovery was accompanied by an increase in total base in two of the three patients for whom figures are available both before and after treatment. Gojeher and his co-workers<sup>16</sup> noted an increase in serum potassium during chronic alcoholism. In combination with a lowered total base this finding would indicate that the serum sodium may be reduced more than the total base determination shows.

Himwich and co-workers,<sup>17</sup> and Nicholson and Taylor,<sup>18</sup> found an increase in blood lactic acid and a fall in carbon dioxide combining power and in blood pH in acute and chronic alcoholism. The fall in carbon dioxide combining power was confirmed in the present study. It is suggested that a contributory factor in the production of the acidosis in some patients is a deficiency in total base. However, the decrease in total base was less than the decrease in chloride and carbon dioxide combining power, with a resultant rise in undetermined anion. An increase in lactic acid or ketonic acids would provide a plausible explanation. Blood lactic acid, pyruvate, and acetoacetic acid determinations were performed in patient 13. Values only slightly above normal were found. The results, together with chloride, bicarbonate, and protein, left an apparent anion deficit of about 10 meq., suggesting the presence of other organic anions.

Examination of the spinal fluid revealed evidence of altered composition. Disregarding the cases with high blood sugars, about one-half of the remainder showed spinal fluid sugar above normal. A large proportion of the spinal fluid protein values were also above normal. The latter finding suggests either injury to the central nervous system or increased capillary permeability. Low spinal fluid chloride values can be explained by diminution in serum chloride. However, in two cases, in spite of a deficiency in serum chloride, spinal fluid chloride was maintained at normal concentration.

#### SUMMARY

1. Fifteen patients with delirium tremens were studied chemically on admission and after recovery.

2. Initially hemoconcentration, hypochloremia, lowered alkaline reserve, and some evidence of a decrease in total base were found. The volume of fluid available for solution of thiocyanate was decreased. Clinical and chemical evidence indicated the presence of dehydration.

3. Recovery was accompanied by a restoration of body fluids and electrolytes to normal composition.

4. Delirium tremens should be treated by methods which promote the restoration of fluid and salt.

5. Changes in the cerebrospinal fluid suggested the occurrence of injury to the central nervous system in certain patients.

I wish to express my appreciation to Dr. J. F. Stouffer and the Psychiatric Staff for permission to study their patients, and to Dr. J. G. Reinhold for suggestions and criticism.

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# THE EFFECT OF THE INTRAVENOUS ADMINISTRATION OF PHOSPHATE SOLUTION IN NORMAL RABBITS

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SHELLING<sup>1</sup> emphasized the importance of phosphates in the successful treatment of animals exposed to the lead hazard and advocated its clinical use. Gray and Greenfield,<sup>2</sup> and later Gant,<sup>3</sup> used the high phosphorus diets clinically in the treatment of patients with industrial plumbism. It was shown experimentally<sup>4, 5</sup> that a high phosphate diet fed to rats during exposure, as well as during deleading, caused a sharp reduction in the amount of lead in the circulating blood. Kowaloff<sup>6</sup> employed a high phosphorus regime in the treatment of a child with lead encephalopathy and showed a marked reduction in the circulating blood lead. In all the cited animal experiments the phosphate was administered orally. The oral administration of any form of therapy in patients with lead encephalopathy may not be practical because of vomiting. Calcium administered to these patients may aggravate the picture since it causes an increase in the circulating blood lead. Under these circumstances, the intravenous administration of a phosphate solution may be a life-saving procedure. Gant<sup>3</sup> employed the alkaline dibasic sodium phosphate solution intravenously in the treatment of plumbism without untoward effects. Sobel<sup>7</sup> and his co-workers have prepared a phosphate solution and used it experimentally in rabbits. They demonstrated that the intravenous administration of their phosphate solution produced a sharp reduction in the blood lead in many experimental animals, in some instances, within one hour after the injection.

The purpose of the following experiments was to observe the behavior of normal animals following the intravenous administration of the phosphate solution prepared by Sobel and his associates. Analyses of the blood and urine were made before and after the injection of the phosphate solution in order to observe the curves of the calcium and phosphorus levels in the blood and in the urine.

The solution used consisted of one part of sodium biphosphate and four parts of sodium phosphate dissolved in 45 parts of distilled water. This made a 10 per cent solution. It was filtered, sterilized, and set up in 10 c.c. ampoules. The pH of the mixture was approximately 7.4 so that no disturbance of the acid-base balance was to be expected following its intravenous administration.

Young chinchilla male rabbits weighing between 2 and 6 pounds were used in these experiments. The animals were anchored on an animal board and catheterized with a No. 6 French ureteral catheter. The marginal veins of both ears were then prepared with alcohol and xylol. After a control specimen of blood was taken from one ear, the phosphate solution was injected into the marginal vein of the opposite ear. The catheter was left in situ, and the urinary drainage was collected in individual containers at hourly intervals so that each

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specimen represented the kidney output for one hour. Samples of blood were obtained from the marginal ear veins at one-hour intervals. Calcium and phosphorus determinations of each of the urine and blood specimens were then made. The animals were kept under observation for four hours. During the experiment the rabbits were fed water by pipette. Food was withheld until after the completion of the experiment.

*Experiment 4.*—Weight of rabbit 2.95 kg.

The control specimens of urine and of blood were obtained. Five cubic centimeters of the phosphate solution were injected slowly. No abnormal behavior was noted. The specimen of urine obtained at the end of the first hour began to show traces of macroscopic blood. Samples of both urine and venous blood were collected at one-hour intervals for four hours following the completion of the injection.

TABLE I

| SPECIMEN | SERUM                     |                              | URINE          |                           |                              |
|----------|---------------------------|------------------------------|----------------|---------------------------|------------------------------|
|          | CALCIUM<br>(MG./100 C.C.) | PHOSPHORUS<br>(MG./100 C.C.) | VOL.<br>(C.C.) | CALCIUM<br>(MG./100 C.C.) | PHOSPHORUS<br>(MG./100 C.C.) |
| Control  | 16.4                      | 4.8                          | 14             | 134.8                     | 20.2                         |
| 1st hour | 9.4                       | 6.8                          | 15             | 108.0                     | 98.5                         |
| 2nd hour | 14.4                      | 5.8                          | 14             | 20.8                      | 48.5                         |
| 3rd hour | 12.9                      | 5.4                          | 9              | 94.8                      | 33.6                         |
| 4th hour | 12.6                      | 5.3                          | 6              | 149.2                     | 49.7                         |

Six experiments in all were performed. Table I represents the typical responses noted. In order to determine whether the blood, which appeared following each injection of phosphate solution, was the result of a sudden precipitation of calcium phosphate crystals, two additional experiments were performed. In the first, the catheter was passed into the bladder and left in situ for four hours. No injection was given this animal. Blood appeared in the specimen obtained during the second hour. The catheter was then withdrawn sufficiently to remove the redundant portion of the catheter which, it was felt, was irritating the wall of the bladder. However, enough was left in the bladder to insure a free flow of urine. The subsequent specimens of urine were clear. In the second control rabbit the catheter was passed into the bladder, and after the sphincter reflex was exhausted and the catheter slipped into the bladder, it was withdrawn so that only enough remained in the bladder to insure a free flow of urine. None of the specimens obtained over the period of four hours contained blood. It was, therefore, felt that the blood noted in the specimens obtained in the previous experiments resulted from the irritation to the bladder wall caused by the catheter.

#### DISCUSSION

All the animals used survived the experiment and were alive for at least six months thereafter. The administration of the phosphate solution to the rabbits caused a sharp rise in the concentration of the serum phosphate as well as in the urine phosphate, and a decrease in the concentration of the serum calcium as well as in the urine calcium. During the second hour there was a secondary rise in the serum and urine calcium levels. The serum and urine calcium values did not return to the control levels within the period during which the animals were kept under observation. It was also noted that the serum and urine phosphate levels showed a secondary decrease, usually beginning within the second hour. Again the recovery fell short of the control level within the

period during which the rabbits were kept under observation. At no time did the observed calcium reach the level which is associated with low calcium tetany. Convulsive seizures were noted in two rabbits and fine tremors in one. These untoward results may have followed the formation of colloidal calcium phosphate, as demonstrated by McLean and Hindrichs<sup>8</sup> in their experimental animals.

Gajato<sup>9</sup> demonstrated that the toxicity of disodium phosphate was three times as great as the toxicity of monosodium phosphate. The pH of the phosphate solution used in the above experiments was 7.4 and closely approximated the pH of the blood. It appears from the experiments that a relatively large amount of this particular phosphate solution (0.463 Gm. per kilogram of body weight) may be administered intravenously. This phosphate mixture does not disturb the acid-base balance as does the disodium phosphate solution when administered intravenously, and, therefore, may be used with greater safety. There are many reports of the intravenous use of phosphate solution in the experimental literature. None have been found in which the phosphate solution was employed as a therapeutic procedure in plumbism. A report of these preliminary experiments was, therefore, considered indicated.

#### SUMMARY

1. A 10 per cent sodium phosphate solution at the pH of plasma was administered intravenously to rabbits.

2. The serum calcium level dropped and the serum phosphorus level rose following the intravenous administration of the phosphate solution.

3. The concentration of the urine calcium dropped and the urine phosphorus increased following the intravenous administration of the phosphate solution.

4. Convulsions were noted in two animals and tremors in one animal used in this experiment.

5. All rabbits were alive six months following the experiment and appeared to be normal.

I wish to thank Dr. S. M. Gordon, of The Endo Products Company, for his cooperation in preparing this solution suitable for intravenous use, and A. E. Sobel, of the Pediatric Research Laboratory of the Brooklyn Jewish Hospital, for his cooperation and many valuable suggestions.

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# LABORATORY METHODS

## GENERAL

### A NOTE ON THE PROBABILITY OF ERROR IN THE DIAGNOSIS OF RABIES BY MICROSCOPIC SEARCH FOR NEGRI BODIES\*

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#### INTRODUCTION

THE microscopic diagnosis of rabies is accomplished by different workers in various ways; some prefer to look for Negri bodies in paraffin sections, some choose to make impressions, and others make smear preparations. Of the variety of methods the microscopic examination of smears or impressions made from Ammon's horn probably holds pride of place by virtue of its simplicity and the rapidity with which it can be performed. In any case, however, it is probable that a certain number of errors in diagnosis occur due to failure to demonstrate the characteristic intracytoplasmic inclusion bodies in the nerve cells.

Negri-Luzzani<sup>1</sup> reported the results of a study of 4,961 brain specimens by microscopic examination checked by animal inoculation; a total of 3,058 were positive by microscopic examination, but 6.7 per cent of the microscopically negative specimens were also found positive on animal inoculation. Koch and Jahn<sup>2</sup> reported a similar study of specimens received at the Robert Koch Institute during the period 1913 to 1928. Of a total of 8,366 specimens examined, 4,682 were positive, and of those negative microscopically, 11.8 per cent were positive when inoculated into animals. Leach<sup>3</sup> has also reported that of 1,032 brain specimens examined, 12 per cent of those reported microscopically negative were positive on mouse inoculation. Commenting on the probability of such errors in diagnosis, Leach says, "the percentage of error will probably vary also with the prevalence of the disease in the geographic areas from which the material for diagnosis is obtained. In regions of high endemicity one might expect a high percentage of Negri negative specimens proving positive on animal inoculation."

When it is necessary to establish beyond question the diagnosis of rabies, animal inoculation is imperative. Until recently the guinea pig and rabbit have been considered the animals of choice for this purpose, but since the demonstration by Hoyt and Jungeblut<sup>4</sup> that the intracerebral injection of rabies

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virus into white mice produced typical and constant infection, the white mouse has become increasingly popular as a test animal.

The only comparative study of the guinea pig and mouse inoculation methods we have found in the literature is that of Willett and Sulkin.<sup>5</sup> These workers reported that of 25 specimens inoculated in parallel into mice and guinea pigs all were positive in the mice, while four were negative in the pigs. Perhaps this is too small a group to be statistically significant, but it does indicate that occasional positive specimens will be missed by guinea pig inoculation.

#### EXPERIMENTAL

With a view of testing particularly the accuracy of the foregoing statements as to errors in microscopic examinations, and of determining the actual percentage of error occurring in the routine diagnostic work in our laboratories, the results detailed here are reported. The sources of the material studied were the diagnostic laboratories of the State Department of Health of Alabama during the years 1937 to 1939, inclusive, and of the Georgia Department of Health during 1937.

In the routine procedure the animal heads were received in the laboratories, the brains were removed, and smears were made and examined in the usual fashion. Sellers' stain was used to demonstrate the Negri bodies in both laboratories. After the smears had been prepared for direct microscopic examination, the remainder of the Ammon's horn was placed in pure glycerin and sent to the Rabies Laboratory for animal inoculation. Here white mice were injected intracerebrally with 0.03 c.c. of a 1:10 aqueous suspension of the hippocampus after trituration with alundum and centrifugation to throw down coarse particles. Table I gives an analysis of the findings on 1,320 specimens from Alabama, and on 754 specimens from Georgia.

TABLE I

NUMBER OF SPECIMENS AND RESULTS OF MICROSCOPIC EXAMINATION AND MOUSE INOCULATION IN ALABAMA FROM 1937 TO 1939 AND IN GEORGIA DURING 1937

| LABORATORY     | SPECIMENS EXAMINED | MICRO. -<br>MOUSE - | MICRO. +<br>MOUSE + | MICRO. -<br>MOUSE + | MICRO. +<br>MOUSE - | MICRO. -<br>MOUSE + | MICRO. +<br>MOUSE - | MICRO. -<br>MOUSE + | MICRO. +<br>MOUSE + |
|----------------|--------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| Alabama, 1937  | 527                | 353                 | 111                 | 51                  | 5                   | 2                   | 4                   | 1                   | 0                   |
| Alabama, 1938  | 588                | 377                 | 125                 | 65                  | 6                   | 9                   | 5                   | 0                   | 1                   |
| Alabama, 1939  | 205                | 167                 | 22                  | 11                  | 0                   | 2                   | 2                   | 0                   | 1                   |
| Alabama, Total | 1320               | 897                 | 258                 | 127                 | 11                  | 13                  | 11                  | 1                   | 2                   |
| Georgia, 1937  | 754                | 445                 | 231                 | 62                  | 6                   | 5                   | 0                   | 4                   | 1                   |

Table I also gives the total number of specimens examined microscopically and subsequently inoculated into mice, together with a complete breakdown of the findings by both methods of diagnosis. Examination of the table brings out two particularly significant points: first, that in both Alabama and Georgia a significant number of specimens are reported as microscopically nega-



tive, which are actually found positive when inoculated into mice; second, that the number of specimens reported positive on the basis of microscopic examination, which are found to be actually negative when injected into mice, is insignificant.

Table II shows the number and per cent of specimens reported microscopically negative but found positive on mouse inoculation, together with the number and per cent of specimens reported microscopically positive but found mouse negative in the Alabama laboratories from 1937 to 1939, inclusive, and in the Georgia laboratories during 1937.

TABLE II

NUMBER AND PER CENT OF SPECIMENS MICROSCOPICALLY NEGATIVE FOUND MOUSE POSITIVE AND THE NUMBER AND PER CENT OF SPECIMENS MICROSCOPICALLY POSITIVE FOUND MOUSE NEGATIVE IN ALABAMA FROM 1937 TO 1939 AND IN GEORGIA DURING 1937

| LABORATORY         | YEAR    | MICRO. NEGATIVE |                  |      | MICRO. POSITIVE |                  |     |
|--------------------|---------|-----------------|------------------|------|-----------------|------------------|-----|
|                    |         | TOTAL SPECIMENS | FOUND MOUSE POS. |      | TOTAL SPECIMENS | FOUND MOUSE NEG. |     |
|                    |         |                 | NUMBER           | %    |                 | NUMBER           | %   |
| Alabama            | 1937    | 404             | 51               | 12.6 | 116             | 5                | 4.1 |
| Alabama            | 1938    | 442             | 65               | 14.7 | 131             | 6                | 4.6 |
| Alabama            | 1939    | 178             | 11               | 6.2  | 22              | 0                | 0.0 |
| Alabama<br>(Total) | 1937-39 | 1024            | 127              | 12.4 | 269             | 11               | 4.1 |
| Georgia            | 1937    | 507             | 62               | 12.2 | 237             | 6                | 2.5 |

From the figures given in Table II it will be noted that addition of the total microscopically negative specimens and microscopically positive specimens for each year gives a sum total somewhat different from the total number examined for that year, as given in Table I. The reason for this apparent discrepancy is that in Alabama in 1937, for example, there were actually only 404 specimens diagnosed as microscopically negative which when inoculated into mice gave a definite positive or negative result. In other words, the 7 specimens shown in Table I in which either the microscopic or mouse result was not clear-cut, i.e., was doubtful, have been eliminated.

## DISCUSSION

It is probable that no single method of microscopic brain examination would yield 100 per cent correct results in the diagnosis of rabies. From the findings presented here it appears that in the laboratories of the Alabama State Department of Health, over a three-year period, slightly over 12 per cent of the specimens reported as negative, on the basis of microscopic examination of stained smears prepared from Ammon's horn, were in fact positive. During 1937 the microscopically negative mouse positive specimens constituted 12.6 per cent of the total diagnosed microscopically negative in Alabama, and 12.2 per cent of the total for the same year in Georgia.

It also appears that errors occur, but to a lesser extent, in the opposite direction. In Alabama, over the three-year period, just about 4 per cent of the specimens diagnosed as microscopically positive were negative when injected into mice. In 1937 in Alabama the number of microscopically positive specimens found negative on inoculation was 4.1 per cent of the total reported micro-

microscopically positive, while in Georgia it was 2.5 per cent. However, these figures lose their apparent significance when the total number of specimens examined is considered.

An attempt at explanation of the above contradictions suggests speculation in several different directions: (1) the certainty with which Negri bodies are recognized by the examiners; (2) the failure to examine smear preparations carefully enough or to make them from the portion of the brain in which Negri bodies were present in sufficient numbers to be detected; (3) the presence in the dog population of strains of the virus which fail to elicit Negri body formation in the dog though capable of giving rise to the disease in mice; (4) the prevalence of distemper or other disease in the dog population which might give rise to inclusion bodies that could be mistaken for Negri bodies; (5) the failure to inject enough virus into the mice to cause rabies though Negri bodies were demonstrable in the original smears; (6) the viability of the virus when injected into mice due to its age or other factors.

#### CONCLUSIONS

1. In an area of high endemicity of rabies errors have been shown to occur in both directions in the microscopic diagnosis of the disease.

2. In Alabama it has been shown that over a three-year period an average of 12.4 per cent of the specimens reported as microscopically negative were actually positive, as shown by mouse inoculation.

3. In Alabama in 1937 the percentage of specimens microscopically negative and found positive on mouse injection was 12.6, while in Georgia the percentage was 12.2 for the same year.

4. In Alabama in 1937 the percentage of microscopically positive specimens found negative on mouse injection was 4.1, while in Georgia it was 2.5.

5. It is not possible to attribute definitely the discrepancies noted above to any one cause.

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# A METHOD FOR PREVENTING BLOOD CLOTTING IN CIRCULATION STUDIES\*

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SINCE the introduction of methods for measuring blood pressure in acute experiments on laboratory animals, the prevention of coagulation has been an annoying problem. Various methods have been proposed to prevent coagulation, and several anticoagulants have been used, some of which, when introduced into the circulation, are apt to produce toxic manifestations and alter the experimental results. MacCracken and Werness<sup>1</sup> proposed a method in which a portion of the carotid artery was isolated and sectioned, and the proximal end was passed through and everted over the end of a short glass tube and tied in place. It was then placed in a small glass chamber filled with sodium sulfate solution. A side arm of this chamber was connected to a mercury manometer. This device avoided the use of a cannula but was somewhat troublesome to set up, and the authors admitted that clots occasionally formed.

Trendelenburg<sup>2</sup> proposed a method in which the anticoagulant solution (one-sixth molar sodium bicarbonate) was introduced into the cannula in a slow stream. The rate of flow was regulated by dropping mercury into a closed vessel containing the anticoagulant solution and connected to the cannula. This method, while satisfactory in some respects, is open to the objection that the anticoagulant solution is continuously introduced directly into the animal.

In using a mercury manometer marked changes in blood pressure result either in a sudden flow of the anticoagulant solution into the animal or in a complete filling of the cannula with unprotected blood. In the former case the anticoagulant may affect the experimental results, and in the latter case a clot may form in the cannula. Even the pressure changes occurring with the heartbeat continually wash a considerable amount of blood in and out of the cannula, rapidly dissipating the anticoagulant. The use of a membrane manometer† greatly reduces the volume of this ebb and flow.

In an attempt to improve the technique of preventing clotting, a new type of arterial cannula, to be used in conjunction with heparin or other anticoagulants, has been devised. The cannula (Fig. 1), essentially an ordinary three-way wash-out cannula, has a fourth short outlet with a slightly flared rim and is placed at right angles to the other three outlets. A rubber serum vial stopper‡ (A) is inserted into the fourth outlet tube, and the thin upper sleeve is inverted over the flared rim of the tube. This securely anchors the stopper. The tip of the cannula is introduced into the artery in the usual manner and is filled with

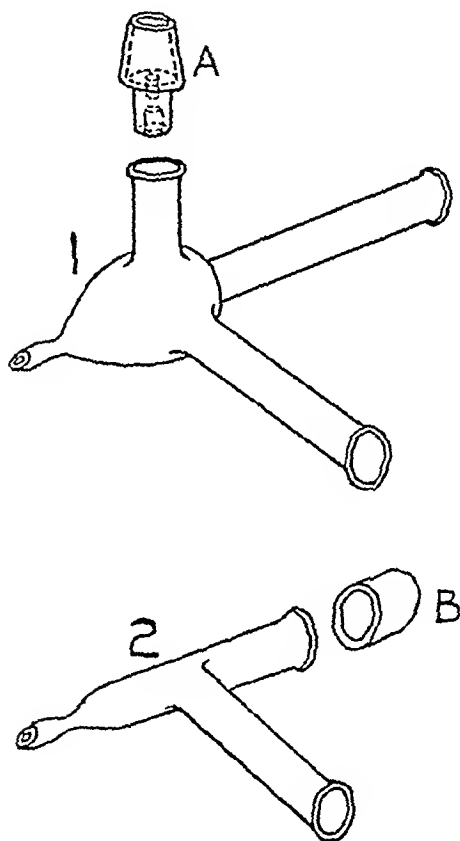
\*From the Scientific Department, Pharmacology Laboratory, Hoffmann-La Roche, Inc., Nutley.

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†The Harvard Apparatus Co., Dover, Mass.

‡No. 1A sleeve stopper, obtainable from the West Company, 1117 Shackamaxon Street, Philadelphia.

normal saline under enough force to wash out all air bubbles through the side arm. The side wash-out arm is closed and the connection to the manometer is made. Pressure in the system is raised to about 150 mm. of mercury. The artery clamp is removed and 0.6 mg. of heparin\* in 0.15 ml. of solution is introduced immediately through the rubber cap by means of a hypodermic needle ( $\frac{1}{2}$  inch, No. 25). In blood pressure studies on cats this dose of heparin is usually repeated one to three times during an experiment. Although the solution in the cannula gradually becomes diluted, even with the membrane manometer, the dilution occurs much more slowly than with a mercury manometer.



Figs. 1 and 2.

Any other anticoagulant can be used, of course, but heparin is preferable, since it is the only physiologically normal anticoagulant known and available. Complete heparinization of an animal requires considerably more heparin than three or four of the above doses. According to Reinert and Winterstein,<sup>3</sup> the clotting time of rabbit's blood is longer than one hour, three hours after the intravenous injection of 3,000 a.c.u. per kilogram, or two hours after the injection of 1,500 a.c.u. per kilogram. From this it is estimated that a 3 kg. animal would require fifteen to thirty times as much heparin for general heparinization lasting three hours as for local use by introduction directly into the arterial cannula.

\*Heparin was employed in the form of Lliquamin 'Roche' containing 2,000 Roche "anti-coagulant units," or 4 mg. per milliliter.

A venous cannula\* (Fig. 2) was designed to facilitate intravenous administrations, particularly when a great many injections have to be made during the course of an experiment. The side arm of the cannula is connected to a reservoir of physiologic saline (a 50 c.c. burette is most convenient). The rubber vial cap† (B) is slipped over the end of the tube. Injections are made, using a syringe and No. 22 needle, preferably long enough to reach just to the constricted tip of the cannula. The solution is injected and immediately washed in with 1 or 2 c.c. of saline. The rubber cap B used with the venous cannula is not satisfactory for the arterial cannula because of the much higher arterial tension, but under venous pressure the cap may be punctured many times without the occurrence of leakage.

#### SUMMARY

The prevention of clotting during a blood pressure experiment requires a suitable antieoagulant, preferably heparin; a membrane manometer to reduce the volume of the ebb and flow of the blood; and a special arterial cannula herein described for introducing small volumes of the antieoagulant solution.

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\*The arterial and venous cannulas were made by the Scientific Glass Apparatus Co., Bloomfield, N. J.

†Size No. 1c gum rubber vial cap also made by the West Company.

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### THE LAUGHLEN TEST FOR SYPHILIS\*

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NUMEROUS reports have been made on the degree of reliability of the Laughlen test. Some have been favorable; others have been adverse.

We wish to report the results of 1,008 consecutive tests on inactivated serum made at the Western State Hospital in comparison with the Kolmer-Wassermann and Kahn tests.

Of the 1,008 specimens, 112 gave reactions of some degree with one or more of the three tests. Most of these patients had syphilis of the central nervous system. Results of 336 tests in these 112 cases were classified as "satisfactory," "questionable," or "unsatisfactory." In the "satisfactory" group all three tests gave positive reactions. Usually, approximately the same strength of reaction occurred with the three tests; however, in many instances a moderate variation occurred. In the "questionable" group a weak reaction occurred with one

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\*From the Division of Pathology, Western State Hospital, Fort Steilacoom.  
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test in the absence of reaction with the other two tests, or there was no reaction with one test in the presence of weak reactions with the other two tests. In the "unsatisfactory" group a strongly positive reaction occurred with one test in the absence of a reaction with the other two tests, or vice versa. It is our purpose not to judge whether, in such instances, the minority or the majority was correct, but simply to compare the tests with each other.

The Kolmer test gave 98 "satisfactory," 8 "questionable," and 6 "unsatisfactory" results. The Kahn test gave 102 "satisfactory," 8 "questionable," and 2 "unsatisfactory" reactions; the Laughlen test gave 106 "satisfactory," 3 "questionable," and 3 "unsatisfactory" results. In two cases in which the Kolmer test was strongly positive in the absence of a reaction to the other two tests, the patients were known to have syphilis; treatment had been instituted. Some of the "unsatisfactory" Kolmer reactions may have been correct, although in the minority.

Judged on the afore-mentioned basis, the Kolmer test showed 99 per cent sensitivity and 91 per cent specificity; the Kahn test showed 98 per cent sensitivity and 93 per cent specificity; the Laughlen test showed 95.5 per cent sensitivity and 99 per cent specificity. "Questionable" reactions were included in computing sensitivity and specificity.

The greater sensitivity of the Kahn test indicates its greater reliability than the Laughlen test as an emergency test for blood donors. If one uses inactivated serum, the Kahn test requires little more time than the Laughlen test.

The Laughlen test appears to be worthy of use with the Kolmer-Wassermann and Kahn tests in providing, without burdening the laboratory unduly, a routine three-test repertoire.

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## A PRESSURE SEITZ FILTER FOR LABORATORY USE\*

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THERE appears to be a need for a small laboratory sterilizing filter capable of handling small volumes of solutions to render them sterile for injection purposes. The usual laboratory filter is dependent upon suction to draw the solution through it and is, therefore, limited in its use for volatile and viscous solutions. In addition, the suction type actually requires some manipulation to seal the solution into a contamination- and evaporation-proof container under sterile conditions. Many existing filters have a tendency to retain comparatively large amounts of solution and, for this reason, it becomes impractical to use such a filter with small volumes. The device described here is designed to eliminate these difficulties and to make it possible to place the sterilized solution directly into a sterile vial which automatically seals itself against contaminants and evaporation.

Pressure is used to force the solution to be sterilized through a Seitz paper mat. The solution is forced directly into a sterile, rubber-closed vial, thereby eliminating all possibilities of contamination.

The apparatus is made of chromium plated brass and is constructed to withstand a pressure of approximately 150 pounds per square inch. Fig. 1 shows the constructional details. A standard Seitz mat is cut to the desired size and placed over the removable perforated disk *C*. The lower end of the apparatus is tapered to fit a standard hypodermic needle. The two main parts are knurled at *A* and *B* to facilitate the tightening of the Seitz mat. The top plug *D* is fitted with a lead gasket to prevent leakage.

### METHOD

The apparatus is assembled, placed in a metal container, and autoclaved for fifteen minutes at 15 pounds' pressure. A vial of an appropriate size to receive the sample and with two hypodermic needles, plugged with cotton and penetrating its rubber cap (see Fig. 2), is autoclaved at the same time. After cooling, one of the cotton plugs is removed, and after flaming the needle, it is attached to the apparatus, and the device is fastened into a support. Since no bacteria can enter the vial, the apparatus may be kept in this condition as long as desired.

Pressure tubing is attached to the air inlet. A high pressure tank of nitrogen or an ordinary compressed air jet may be used to supply the required pressure.

\*From the Department of Biochemistry, the University of Chicago.  
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The sample (1.5 to 10 c.c.) is placed into the apparatus by removing cap *D*. The cap is tightened by means of a rod inserted through its top. Pressure is applied slowly at first, and finally to a point just sufficient to force the liquid into the vial. When the desired sample is obtained, the pressure is released and the needles are removed from the vial.

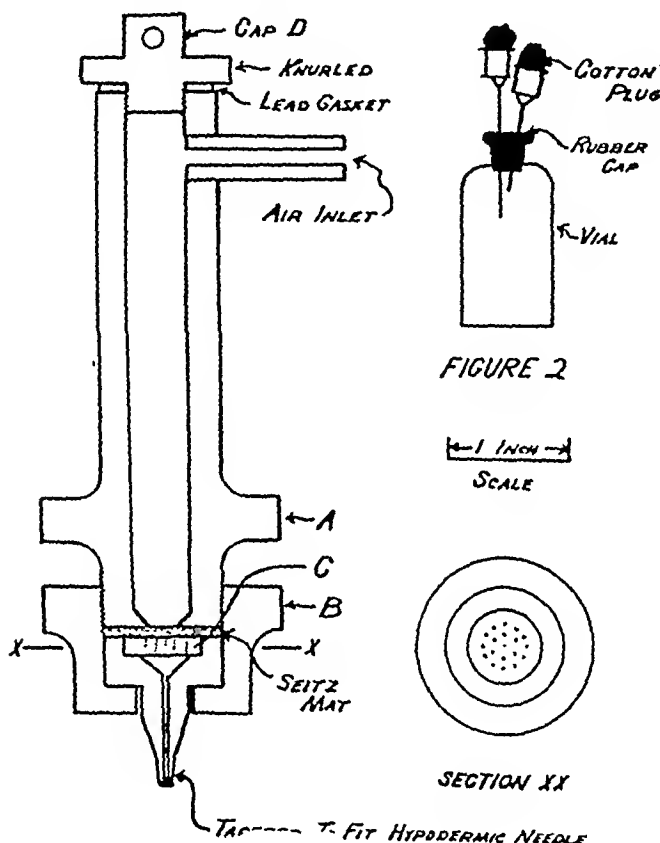


Fig. 1.

The apparatus was tested using a culture of *Bacterium prodigiosum* in nutrient broth. The culture was passed through the filter, and the filter and part of the original culture were plated out. Similar tests were performed using bacteria in solutions passed through the filter.

The apparatus may be secured from the machine shop, of the Department of Biochemistry, the University of Chicago.



## MESTER'S TEST IN RHEUMATOID ARTHRITIS AND SPONDYLITIS RHIZOMELIQUE\*

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IN 1937 Mester<sup>1</sup> reported results of a salicylic acid skin test in patients with rheumatic and nonrheumatic conditions and concluded that it was a specific diagnostic test for "rheumatic diseases." This conclusion was confirmed in similar studies by Lenoeh,<sup>2</sup> and later by Braghin.<sup>3</sup> The latter author reported that the test was positive in all 45 patients who were suffering from rheumatic diseases, including "acute, subacute, and chronic rheumatic polyarthritides, rheumatic ankylopoietic spondylarthritides, rheumatic ischialgia, rheumatic fever with cardiac complications, and chronic rheumatic iridocyclitis." The test was negative in patients with "non-rheumatic" joint diseases, which included gonococcal arthritis, gout, tuberculosis, and syphilis.

If the test were sensitive and specific, as stated by the above-mentioned authors, it would be of great value in the diagnosis of early spondylitis rhizomelique and of atypical atrophic arthritis, and in the exclusion of gout, gonococcal arthritis, and degenerative disease of joints. Since it is sometimes difficult to differentiate these forms of rheumatism, we desired to determine the dependability of Mester's test.

Employing the technique of Braghin,<sup>3</sup> the test was performed in 20 patients with rheumatic disease, 16 of whom had characteristic rheumatoid arthritis, and 4 had spondylitis rhizomelique, and in a control group of 10 nonrheumatic patients. Blood was withdrawn from the middle finger of the right hand of the fasting patient before, and again thirty and sixty minutes after, the administration of two intrautaneous injections of 0.2 c.c. of a sterile 0.1 per cent aqueous solution of salicylic acid, and the number of leucocytes in each blood specimen was determined. The injections were made on the volar surface of the right forearm at a distance of about 5 cm. from each other. These injections were usually followed by pain and burning of short duration, and by the formation, at the sites of injection, of erythematous wheals which disappeared in a few hours. A positive test consists of a transient diminution of the leucocytes, usually within the first thirty minutes, and less frequently within the second thirty minutes after the injection of the salicylic acid solution. A decrease in the leucocyte count of 20 per cent or more was considered a positive test; a rise in the leucocyte count, no change or fall of less than 20 per cent, were considered as negative.

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The test gave negative results in all 10 patients with nonrheumatic disease. In the group of 20 with rheumatoid arthritis or spondylitis rhizomelique, a positive test was obtained in only 5 instances (Table I).

RESULTS OF MESTER'S TEST IN PATIENTS WITH RHEUMATOID ARTHRITIS AND SPONDYLITIS RHIZOMELIQUE

| DIAGNOSIS                | LEUCOCYTE COUNT (CELLS PER C.M.M.) |                         |                         |          |
|--------------------------|------------------------------------|-------------------------|-------------------------|----------|
|                          | BEFORE INJECTION                   | 30 MIN. AFTER INJECTION | 60 MIN. AFTER INJECTION | RESULT   |
| Rheumatoid arthritis     | 10,600                             | 6,100                   | 5,200                   | Positive |
| Rheumatoid arthritis     | 8,850                              | 8,950                   | 9,000                   | Negative |
| Rheumatoid arthritis     | 6,400                              | 6,200                   | 6,200                   | Negative |
| Rheumatoid arthritis     | 7,250                              | 8,100                   | 8,000                   | Negative |
| Rheumatoid arthritis     | 8,800                              | 9,000                   | 8,700                   | Negative |
| Rheumatoid arthritis     | 7,000                              | 7,200                   | 6,900                   | Negative |
| Rheumatoid arthritis     | 7,950                              | 7,700                   | 7,400                   | Negative |
| Rheumatoid arthritis     | 4,100                              | 4,200                   | 4,050                   | Negative |
| Rheumatoid arthritis     | 8,850                              | 5,100                   | 5,400                   | Positive |
| Rheumatoid arthritis     | 4,000                              | 4,200                   | 4,300                   | Negative |
| Rheumatoid arthritis     | 8,100                              | 6,900                   | 8,200                   | Negative |
| Rheumatoid arthritis     | 10,450                             | 10,600                  | 10,400                  | Negative |
| Rheumatoid arthritis     | 6,750                              | 6,200                   | 6,400                   | Negative |
| Rheumatoid arthritis     | 7,200                              | 6,400                   | 6,000                   | Negative |
| Rheumatoid arthritis     | 8,800                              | 8,450                   | 8,400                   | Negative |
| Rheumatoid arthritis     | 8,300                              | 6,150                   | 8,450                   | Positive |
| Spondylitis rhizomelique | 9,200                              | 9,000                   | 6,100                   | Positive |
| Spondylitis rhizomelique | 4,550                              | 4,100                   | 4,350                   | Negative |
| Spondylitis rhizomelique | 11,300                             | 8,400                   | 8,800                   | Positive |
| Spondylitis rhizomelique | 4,600                              | 4,600                   | 4,450                   | Negative |

Although the test was negative in our control cases, it was also negative in the majority of patients with typical arthritis; in this our results do not agree with those reported by others.<sup>1-3</sup> Braghin, for instance, stated the test was positive in *all* of 45 patients suffering from "rheumatic disease." In our group of 20 patients the test was positive in only 25 per cent. Because of these disappointing results, we did not attempt to determine the "specificity" of the test in differentiating certain rheumatic diseases from gout, gonococcal, and tuberculous arthritis, for even if it were always negative in the latter group, the test would be very unreliable, due to the fact that we found it negative in the majority of patients with characteristic rheumatoid arthritis.

#### CONCLUSION

Mester's test was found to be grossly unreliable as an aid in the diagnosis of characteristic rheumatoid arthritis and spondylitis rhizomelique, which accordingly makes it of little value in differentiating certain forms of rheumatic disease.

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## DETERIORATION OF CORN GERM STEROL\*

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IN 1932 Eagle<sup>1</sup> introduced a new sterol, corn germ sterol, for use in fortifying the antigen used in his Eagle flocculation test for syphilis, in addition to the more widely used cholesterol. The major advantage of this sterol as an antigen sensitizer is that the antigen emulsion for use in the test is more stable if corn germ sterol is used than are emulsions made with antigens sensitized with cholesterol alone. In a later modification of the flocculation test<sup>2</sup> the use of both sterols was again recommended.

Recently four preparations of Eagle antigen made in our laboratories from three different lot numbers of Difco beef heart have been found unsatisfactory for use when fortified with old corn germ sterol and fresh cholesterol. These unsatisfactory antigens when tested with sera, which gave a negative reaction to three other nationally recognized tests, show a markedly granular appearance and do not give the "silky swirl" on shaking that is so characteristic in the negative Eagle test. When read microscopically, the granularity observed would justify a reading of doubtful. Strongly positive sera gave characteristic reactions with these antigens, but weak reactions are difficult, if not impossible, to read accurately.

These four unsatisfactory antigens were prepared, one in March, 1940, two in July, 1940, from two different lots of beef heart, and one in August, 1940, using the same beef heart as was used for one of the July preparations. Judging by the degree of granularity observed in tests with negative sera, the deterioration had progressed very rapidly during these five months. This rapidly progressive deterioration is suggestive of an autocatalytic oxidation.

Separate aliquots of three of the four Eagle antigens have been fortified with the same fresh cholesterol and fresh corn germ sterol. With all three of these antigens the negative sera tested have given a characteristic appearance.

The old corn germ sterol was purchased in December, 1932. Difco Laboratories has informed our laboratories that this sterol was made in 1931. Since its purchase this sterol has been kept at room temperature in the brown glass bottle, stoppered with the paper covered cork as received. At this time the old sterol has a yellow tinge and the fresh sterol is white. This color change is also suggestive of a rancidity oxidation. The melting point of this old sterol has been checked at the Difco Laboratories, and it is found to be about 96° C. over a range of approximately 5° C., while in 1931 when the material was prepared the melting point was sharp at 138° C.<sup>3</sup> Eagle antigen prepared in May, 1938, in which this old sterol was used, is still entirely satisfactory.

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From these observations it can be concluded that corn germ sterol deteriorates with age, undergoing an atmospheric oxidation, and is then unsatisfactory for use in Eagle flocculation antigens. It is suggested that this deterioration be guarded against in laboratories in which the Eagle test is used. It seems advisable to keep this sterol in a refrigerator, since this would tend to slow up oxidation. The one sample of sterol here studied was satisfactory for about six years. Other samples might vary in speed of deterioration, and varying laboratory conditions might markedly affect the rate of this chemical change. It, therefore, seems inadvisable to use corn germ sterol for an extended period of time.

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### FACTORS INFLUENCING THE DEMONSTRATION OF TUBERCLE BACILLI BY CONCENTRATION METHODS\*

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FOR the detection of small numbers of tubercle bacilli in sputum or gastric aspirations the most widely used methods have been culture or animal inoculation. Each of these methods is quite time-consuming, both from the standpoint of the technique employed in the laboratory and the time interval before the clinician receives his report. In addition to this, animal inoculation, which is the more dependable test, is very expensive when used on a large scale. With the increasing use of the laboratory for determining the infectivity of early and convalescent cases of tuberculosis, much study has been expended to determine a method of direct examination which will suffice in a large proportion of such cases and will thus reduce the number of guinea pigs required. A number of methods for concentrating sputum and gastric aspiration have been developed with varying claims for their usefulness. Some investigators have regarded their concentration methods to be superior to guinea pig inoculation. However, it must be remembered that the pathogenicity of an acid-fast organism cannot be determined by the morphology and staining reaction of the organism, and in doubtful cases the guinea pig inoculation is still the final test.

The concentration method depends on (1) the digestion of the mucus and cellular elements in the specimen, and (2) the collection of the organism for staining. The wide variation in the character of the sputum or gastric aspirations leads to difficulty in selecting a digester which will work equally well with all types of specimen. In evaluating the various methods of concentration it

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is necessary to balance the results against the time and expense of procedures. The ideal method to be applied to large numbers of examinations should be simple and should produce a material which is entirely satisfactory for smearing and staining. The concentration should be sufficiently effective so that the material from a large amount of the original specimen can be examined.

The methods for concentrating sputa which are most widely used are the anti-formin method of Uhlenhuth and Xylander<sup>7</sup> and the sodium hydroxide method of Petroff.<sup>4</sup> These methods consist of the digesting of the specimen by alkali, centrifuging out the organisms, and making stained smears. To facilitate the separation of the organisms from the digested specimen, flotation with ligroine or other hydrocarbons, as developed by Kinyoun,<sup>3</sup> or precipitation with alum or ferric chloride (Hanks, Clark, and Feldman<sup>2</sup>) have been used. Volatile hydrocarbons were used by Andrus and MacMahon<sup>1</sup> to aid in sedimenting the organisms. Various modifications of these methods have been reported. A number of other types of digesters have more recently been advocated by various workers who attribute to them definite advantages over normal sodium hydroxide. Among these are such vegetable ferments as caroid (Sullivan and Sears<sup>6</sup>), animal ferments as trypsin (Vogt, Zapposodi, and Long<sup>5</sup>), and organic solvents as "tergitol, penetrant 08" (Petroff and Schain<sup>5</sup>). Each of these is more expensive and less available, and their use is more time-consuming than treatment with normal sodium hydroxide. Therefore it would seem that there should be quite an increase in positive results to make them superior to sodium hydroxide for routine use. The same may be said for the various methods for collecting organisms from the digested specimens.

For these reasons, we have made a study of these various methods in comparison with sodium hydroxide. Our chief concern was to find the best method for handling gastric aspirations, but since sputa are more readily available in quantity, the first part of our study was made using sputa, especially mucoid or mucopurulent types, and the methods giving best results were later applied to gastric specimens.

#### EXPERIMENTAL

The two stages in the concentration of a sputum are: (1) the digestion of mucus and cellular elements, (2) the collection of the organisms for staining.

*Digestion.*—The technique which we used with sodium hydroxide, and with which we compared other methods, was as follows: To a measured quantity of sputum was added an equal quantity of normal sodium hydroxide and this was shaken well. The mixture was incubated for thirty minutes at 37° C. with occasional shaking. At the end of the incubation period the specimen was shaken well, neutralized to just alkaline to bromthymol blue, and centrifuged for thirty minutes at about 2,000 r.p.m.

Sodium hydroxide has the disadvantage of being injurious to the organisms if left too long in contact with them and may destroy their acid-fast character. It also appears to cause some clumping of the organisms so that distribution on the smear may be irregular. These, and the presence of crystals on the smears, were the disadvantages we tried to overcome.

1. *Alkali.* Other alkalies were tried—calcium hydroxide and ammonium hydroxide—using the same technique. A saturated calcium hydroxide solution

required much longer to be effective, and the results then were not equal to those with normal sodium hydroxide. Normal ammonium hydroxide appeared to liquefy the specimen almost as rapidly as normal sodium hydroxide, but it was in no way superior.

2. *Acids.* Acid digestion with 6 per cent sulfuric acid was tried, using the same technique as above, neutralized with sodium hydroxide and with ammonium hydroxide. This was fairly satisfactory for digesting mucus, although not so rapid in action as normal sodium hydroxide. However, pus cells were left intact, and in mucopurulent specimens the sediment after centrifuging was quite heavy. The smears after this treatment show a nice distribution of the organisms, but sometimes they show extraneous material which is difficult to decolorize.

3. *Animal ferments.* Trypsin is an animal ferment which acts on mucus. However, its action is not sufficiently vigorous to take care of all types of specimens and frequently there is a large volume of sediment or quite a viscous digest.

4. *Vegetable ferments.* Caroid, while it does not digest pus actively, is more satisfactory than trypsin. It appears to be more active in an alkaline medium, hence the addition of equal quantities of a 0.1 to 0.2 per cent sodium hydroxide solution to a 5 or 2 per cent caroid solution is more effective than either would be alone. Occasionally caroid smears are difficult to decolorize, but generally the smears have an even background and good distribution of well-stained organisms. Caroid is less destructive to the organisms than sodium hydroxide, and the digest may be allowed to stand several hours without apparent effect on the staining of the acid-fast organisms.

5. *Organic solvents.* "Tergitol, penetrant OS" in solution with sodium hydroxide, as recommended by Petroff, is an active solvent, liquefying sputum in about the same length of time as normal sodium hydroxide. It appears to be more destructive to the organisms, since fewer organisms were found in the smears from the same specimens after this method of digestion than after digestion with normal sodium hydroxide.

From the study made it appears that the most satisfactory digesters are normal sodium hydroxide and caroid (2 to 5 per cent caroid with 0.2 to 0.1 per cent sodium hydroxide). Normal sodium hydroxide has the disadvantage of being injurious to the organisms, and if left too long in contact will destroy the acid-fast properties. However, there is a wide margin of safety between the time needed for satisfactory liquefaction of the specimen and the time at which injury to the organism is evident. Thirty minutes is sufficient time for liquefaction of all types of specimens except the very tenacious or very purulent. When these types of specimens are mixed with one or two volumes of distilled water, shaken well, and then mixed with an equal volume of normal sodium hydroxide, they, also, are generally liquid in thirty minutes. Reduction in the number of organisms demonstrable by smear is not noticed until more than one hour exposure to normal sodium hydroxide.

Caroid is less destructive to the organisms than sodium hydroxide, and the sediment generally makes very nice smears with well-stained organisms. However, since it does not digest the cellular elements there is frequently a heavy sediment, and sometimes the smears are difficult to decolorize.

*Collection of Organisms.*—After the specimen is thoroughly liquefied, the organisms are to be collected for staining. In the above studies this was done by centrifugation for thirty minutes at about 2,000 r.p.m. In addition to this, various methods of facilitating sedimentation were compared and a study made of the factors which might influence the separation of the organisms from the digest.

1. To study the effect of specific gravity, aliquots of the digest were centrifuged without dilution, diluted with two volumes of distilled  $H_2O$ , and with two volumes of 95 per cent alcohol, respectively. These were centrifuged as above. A comparison of diluted specimens with undiluted specimens gave the following results: Of 20 specimens digested with normal sodium hydroxide, 9 showed more organisms without dilution with water than with dilution, 8 showed more organisms when diluted, and 3 were approximately equal. No definite relationship between type of specimen and the effect of dilution could be discovered. Of 10 specimens diluted with alcohol, 7 showed fewer organisms when diluted, and 3 showed about equal numbers. Since 95 per cent alcohol frequently gives a heavy, rather tough sediment, 50 to 75 per cent alcohol was used instead, or 95 per cent in quantities just short of the amount which would produce heavy precipitation. No definite improvement in number of organisms was obtained.

Of 20 specimens digested with caroid (1, 2, or 5 per cent), 12 showed fewer organisms when diluted with two volumes of distilled water, 6 showed more organisms, and 2 were about equal. Of these same specimens 13 showed fewer organisms when diluted with two volumes of alcohol, 6 showed more, and one was about equal. The above results would indicate that the lowering of the specific gravity by dilution with distilled water or alcohol has no great advantage if the specimen is thoroughly liquefied by the digesting process. However, with specimens which give a viscous digest there appears to be an advantage in dilution. It is more satisfactory to add distilled water (equal volume or more, depending on the degree of viscosity) to the specimen and mix it well before the digester is added. This gives a more satisfactory liquefaction with a lower concentration of the digester; for example, 10 c.c. sputum + 10 c.c. distilled water + 20 c.c. normal sodium hydroxide = a concentration of 2 per cent sodium hydroxide. This gives a more satisfactory digest than 10 c.c. sputum + 20 c.c. normal sodium hydroxide which gives a slightly higher concentration of sodium hydroxide.

2. *Chemical flocculation.* The use of alum, ferric chloride, or sodium carbonate to collect the organisms by flocculation of the proteins was of no definite advantage in our hands. The heavy precipitate from a given quantity of digest greatly reduces the amount of the original specimen which can be used for the examination so that any increase in the proportion of organisms precipitated out is balanced by the smaller quantity examined.

3. *Volatile hydrocarbons.* Following the work of Andrus and MacMahon, specimens, after digestion and neutralization, were shaken with a few drops of chloroform (about 1 drop per cubic centimeter of digest) and centrifuged. The chloroform collects in the bottom of the tube, and the sediment containing the tubercle bacilli forms a layer just above it. This sediment forms a satis-

factory material for staining, giving a good distribution of well-stained organisms. Although the results, again, were not entirely consistent, there appeared to be an advantage in the use of chloroform with digested sputa.

Of 36 specimens digested with sodium hydroxide, 22 showed more organisms when treated with chloroform, 10 showed more organisms untreated, and 4 showed about equal numbers. Of 14 specimens digested with caroid, 7 showed more organisms treated with chloroform, 5 showed fewer, and two showed about equal numbers.

#### STUDY OF FACTORS INFLUENCING SEDIMENTATION OF TUBERCLE BACILLI

1. *Surface tension depressors.* On the possibility that a lowering of the surface tension of the suspending medium might allow the organisms to be more readily centrifuged, various materials were added to the digested specimen for the purpose of lowering surface tension. These included a stock soap solution, aerosol (0.1 per cent), sodium glycocholate (5 per cent), urea (5 and 20 per cent), and drefl (0.1 and 1 per cent). The results, as with attempts to lower specific gravity, were so varied and inconsistent that no definite conclusions could be drawn. A study of surface tension values was made together with pH values as given below.

2. *Variations in the acids used for neutralizing and a study of pH values.* Sodium hydroxide digests of sputa were used entirely for this part of the work. In the beginning hydrochloric acid was used for neutralization, but since this gave rather heavy salt precipitates the effects of neutralization with sulfuric acid, oxalic acid, and acetic acid were studied. Comparisons were also made of the number of positive slides and the number of organisms per slide found with the sediment from each. The specimens neutralized with acetic acid gave somewhat better results in the number of organisms found and also gave a smear which was easier to examine, showing less extraneous acid-fast material. In the later studies the sodium hydroxide digests were neutralized with 25 per cent acetic acid.

The pH reading and surface tension readings were made on a number of specimens and compared with the count of bacilli on stained smears to determine if there might be a direct relationship between these values and the separation of organisms from the digest. Each specimen was divided into equal parts, and acid was added to secure various degrees of alkalinity or acidity. Other portions of the specimen were treated with alcohol, tergitol, soap, and aerosol to vary the surface tension. Table I shows the pH and surface tension readings of the preparation of each specimen which showed the largest number of bacilli by smear. The smears were made by spreading the material evenly between two slides, and examination consisted of careful observation of three lines across the slide. In some cases more than one preparation gave approximately equal numbers of organisms. In such cases more than one preparation from a specimen is included in the table.

It is noted that there is a wide range of pH and surface tension values through which sedimentation of the organisms goes on equally well. The optimum pH seems to lie between 9 and 11, while the optimum surface tension is between 63 and 73, which is approximately the reading of the neutralized



sodium hydroxide digest without any further treatment. A cavoid digest of the average sputum is also in this range. These surface tension readings were made on the Du Nouy apparatus and are based on a reading for distilled water of 108. pH readings were made on the Coleman pH electrometer.

The character of the sputa from patients with tuberculosis or suspected tuberculosis varies to such a degree that we have been unable to lay down a method of examination which is definitely superior for all types of sputum.

TABLE I

| SPECIMEN<br>NUMBER | NUMBER OF ORGANISMS     | pH    | SURFACE TENSION |
|--------------------|-------------------------|-------|-----------------|
| 28                 | 88                      | 12.2  | 61              |
| 29                 | 8                       | 10.0  |                 |
|                    | 8                       | 11.8  |                 |
| 30                 | 1                       | 5.7   |                 |
|                    | 1                       | 8.1   |                 |
| 31                 | 1                       | 8.8   | 63              |
|                    | 1                       | 8.8   | 67.5            |
| 32                 | 4.6 (average per field) | 10.15 | 73              |
|                    | 4.6 (average per field) | 9.0   | 70              |
|                    | 4.3 (average per field) | 5.3   | 61              |
| 33                 | 2                       | 9.8   | 60.5            |
|                    | 2                       | 10.75 | 75              |
| 34                 | 6                       | 11.0  | 52              |
| 35                 | 4                       | 9.0   | 67              |
|                    | 4                       | 11.1  | 67              |
| 36                 | 156                     | 8.9   | 73              |
| 37                 | 3                       | 9.15  | 67              |

The inconsistency of results would suggest that the method of handling is less important than the uneven distribution of organisms in the original specimen. To overcome this we believe that a large quantity of the specimen should be digested. The entire quantity should be digested rather than 5 to 10 c.c., as has been recommended for sputa, or rather than the sediment from centrifuged gastric aspiration. After thorough liquefaction long centrifugation is necessary to sediment the bacilli. The addition of chloroform appears to be an aid in sedimentation, without increasing the amount of sediment. On the other hand, chemical flocculation increases the amount of sediment to such a degree that the sediment from a smaller proportion of sputum is used for the smears and the chance of finding bacilli in the smears is not increased.

*Application of Gastric Aspirations.*—A total of 287 gastric specimens, which were negative by direct centrifugation were examined by concentration methods. The quantity used varied from 10 to 40 c.c. At first these were digested with equal quantities of normal sodium hydroxide, but it was discovered that 2 per cent sodium hydroxide was satisfactory for these as the proportion of mucus is generally much lower than in sputa. They were digested for thirty minutes at 37° C., neutralized to approximately pH 9 with 25 per cent acetic acid, and centrifuged for thirty minutes at about 2,000 r.p.m. Of the 287, 34 per cent were positive by one method. In 64 specimens an equal portion was shaken with chloroform before centrifugation. Of these 64 specimens examined both with and without chloroform, 25 were positive with chloroform and 23 without chloroform. The advantage of chloroform was less marked here

than in sputa, perhaps because the gastric specimen is more completely liquefied and has a lower specific gravity than the sputum. The small increase in positive results does not appear to warrant the extra handling.

*Routine Use in the Laboratory.*—For a period of nine months we have been using this method of concentration on routine gastric aspirations and sputum specimens in this laboratory. Letters were sent to the various sanatoriums in the state requesting that, whenever possible, specimens which would ordinarily be sent to this laboratory for guinea pig inoculation should be sent rather for concentration. If this proved negative a second specimen should be sent for guinea pig inoculation. We did not make direct smears as the sanatoriums generally send in only specimens from patients who have been previously negative by smear.

Gastric aspirations were received in the laboratory as either single or pooled specimens (specimens taken on three successive days and pooled) in quantities of from 10 to 100 c.c. The entire quantity was shaken with an equal quantity of 2 per cent sodium hydroxide and incubated at 37° C. for thirty minutes. These were then neutralized with 25 per cent acetic acid to just alkaline to bromthymol blue (pH 8 to 9), and centrifuged at about 2,000 r.p.m. for thirty minutes. In most cases the entire quantity of sediment could be smeared between two slides without making the smears too heavy for examination. These smears were stained by the Ziehl-Neelsen method.

Sputa, unless rather tenacious, were handled in the same way. Those with a large proportion of mucus or pus were first shaken with an equal quantity of distilled water and then with an equal quantity of 2 per cent sodium hydroxide, or if necessary normal sodium hydroxide.

During this period, 807 gastric aspirations and 290 sputa, or a total of 1,097 specimens have been examined. Of these 31.7 per cent of the gastric aspirations and 30.6 per cent of the sputa have been positive by the concentration method; 2.8 per cent of the gastric specimens and 1.3 per cent of the sputa were reported as having atypical acid-fast bacilli present.

One hundred and fifty-four specimens from patients who had received negative reports by concentration on previous specimens were injected into guinea pigs. More than half of these were pooled specimens. Two guinea pigs were injected in such cases. Of the 154 specimens, 89 per cent were negative by guinea pig inoculation and 11 per cent were positive. Of this 11 per cent (17 specimens), 4.5 per cent (7 specimens) were positive in one guinea pig and negative in the other, indicating that the organisms were very few in number. We believe that the positive results gained by careful examination of the concentrated specimens more than compensates for the time required for preparing the specimens and examining the slides, since tubercle bacilli can be demonstrated immediately in many specimens which would otherwise require guinea pig inoculation and a delay of six weeks before the bacilli could be demonstrated.

#### CONCLUSIONS

1. The wide variation in results obtained through the use of various methods of digesting sputa and gastric specimens and precipitating the tubercle bacilli from the digests indicates that the newer methods of handling have lit-

tle, if any, advantage over digestion with normal sodium hydroxide (2 per cent sodium hydroxide with gastric specimens or less mucoid sputa) and long centrifugation.

2. Comparable results in precipitating the organisms can be obtained through a fairly wide range of pH values (9 to 11) and surface tension readings (63 to 73).

3. Dilution with distilled water or alcohol to lower specific gravity was of no value except in the case of very viscous specimens. In such cases dilution with distilled water before digestion aids in liquefaction, and it is not necessary to increase the concentration of the digester.

4. The use of a large amount of specimen, thorough liquefaction and long centrifugation, thus securing a great concentration of the solids, are the essentials of a good concentration method.

We would like to express our appreciation to Dr. W. H. Oatway, Jr., of the Wisconsin General Hospital and to the physicians of various sanatoriums in the state for furnishing us with specimens.

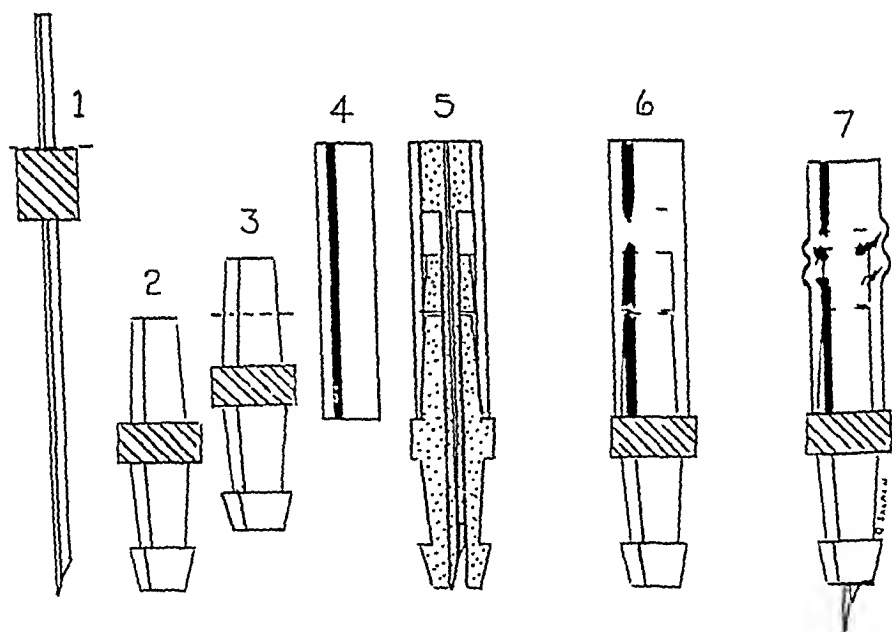
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# A BLOOD LANCET ASSEMBLED FROM COMMON LABORATORY ACCESSORIES\*

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**B**LOOD lancets, because of their size and constant use, are easily lost or misplaced. Internes on laboratory service are notorious for losing them. Consequently, the usual substitute for a lancet is an ordinary needle stuck through a cork. Even though such instruments are effective, it must be granted that the depth to which the needle will penetrate is not readily controllable. In addition, such an instrument often shocks the finer sensibilities of many patients. Certainly, it does not look like an instrument of precision which is expected of the medical profession.



Figs. 1-7.—1, New York Board of Health bleeding needle, gauge No. 19. 2, Needle adapter for ends of rubber tubing. 3, A second adapter to be cut at the dotted line. 4, Rubber tubing as employed for blood counting pipettes. 5, Cross section of the assembled parts. 6, Normal appearance of the instrument. 7, Shows the needle point protruded beyond the shield during action.

A practical lancet can be easily assembled from the following parts: An ordinary blood-letting needle of the New York Board of Health Type, No. 19 gauge, and  $1\frac{1}{4}$  inches long (Fig. 1); two needle adapters for ends of rubber tubing (Fig. 2 and Fig. 3); and a short piece of rubber tubing of the size used

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on blood counting pipettes (Fig. 4). The blunt end of the needle (Fig. 1) and one of the adapters (Fig. 3) are cut off at the dotted lines. All parts are assembled and held together by the rubber tubing, as shown in Fig. 5. This figure shows the lancet in cross section with the needle in its normally retracted position. Fig. 6 shows the completed instrument, while Fig. 7 demonstrates the projection of the needle point. The rubber tubing serves a double purpose of holding the parts together and of acting as a spring for the retraction of the needle point.

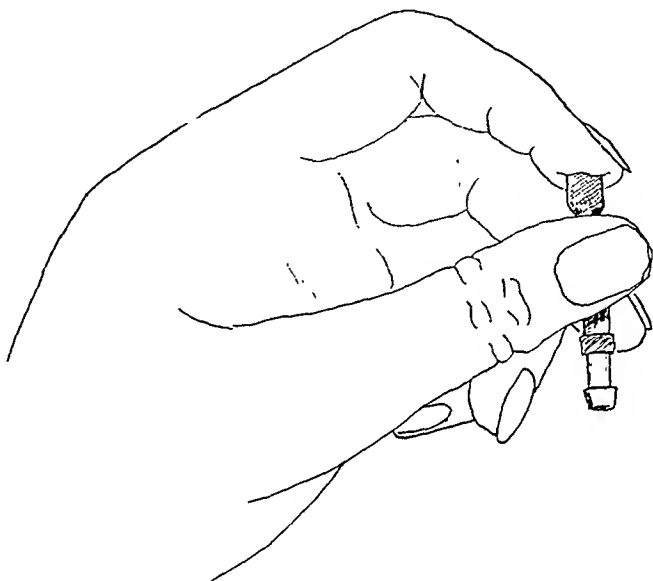


Fig. 8.—The comparative size and position of the instrument.

No dimensions are given, since all parts are of standard size. The only variable is the small section of the adapter in Fig. 3. The length of this section depends on the length of the needle available, so that the latter may extend beyond its normal position to the desired distance when it is penetrating the patient's skin. Fig. 8 shows the relative size of the finished instrument.

The lancet is used by holding it between the thumb and middle finger, as shown in Fig. 8. While one end is resting on the patient's skin, the other is given a quick tap with the index finger. This maneuver will propel the needle beyond its shield to the predetermined depth. The resiliency of the rubber tubing will return the needle to its former protected position.

The lancet has been used for some time and has proved entirely satisfactory.

## A MICROMETHOD OF THE WELTMANN REACTION\*

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THE serum coagulation test, as introduced by Weltmann<sup>1,2</sup> in 1930, is a valuable aid for the differentiation between exudative, inflammatory, or necrotic processes, and fibrotic, proliferative conditions. The value of this reaction was confirmed by many German investigators.<sup>3-5</sup>

Recently, in this country, Levinson and associates<sup>6</sup> carried out a study of the Weltmann reaction on a large series of various diseases as pneumonia, pleuritis, cirrhosis of the liver, sepsis, and especially endocarditis. Their findings confirmed the earlier reports of the worth of this test.

In a second paper Levinson and Klein<sup>10</sup> demonstrated the importance of this test in distinguishing between exudative and proliferative types of lung tuberculosis. They say: "in tuberculosis the Weltmann coagulation test is of importance, because it can be used as a guide in the course of, and the prognosis of the disease. It reflects the tissue changes in the body." However, they write: "When exudative and proliferative changes occur in the lungs at the same time, as happens frequently in tuberculosis, the Weltmann reaction may not be of great assistance. The two divergent activities occurring at the same time result in the coagulation band, striking the normal zone." Although, the standard technique is described in the above-mentioned articles,<sup>9, 10</sup> we shall repeat the description of the original method.

A 10 per cent stock solution of calcium chloride ( $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ ) is prepared. From this solution various dilutions are made, ranging from 0.1 per cent to 0.01 per cent. These solutions are numbered from 1 to 10, beginning with the strongest concentration. Small test tubes, similar to those used for the Wassermann reaction, are placed in a metal rack. These tubes are also numbered from 1 to 10. Five cubic centimeters of the similarly numbered calcium chloride solution are placed in each test tube. To each tube is added 0.1 c.c. of unhemolyzed serum. The solutions are thoroughly mixed, and the tubes are placed in a hot water bath and boiled for fifteen minutes, after which time the tubes are examined. The tube with the solution of highest dilution, at which flocculation is present, is noted. Weltmann called the concentrations at which flocculation occurs as the coagulation band, and designated it as K.B. Normal K.B. is given to the reaction if there is flocculation up to and including the sixth tube. Other workers included the seventh tube as normal.

The findings of various investigators reveal that exudative and necrotic processes as pneumonia, pleuritis, caseous type of lung tuberculosis, recent coro-

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nary thrombosis, lung abscess, and other such conditions cause a narrowing of the K.B. (a shift to the left), or in very severe cases, an absence of any coagulation.

TABLE I

COMPARISON OF THE CONCENTRATIONS BETWEEN THE ORIGINAL METHOD AND THE MICROMETHOD

| <div style="display: flex; flex-direction: column; align-items: center;"> <div style="writing-mode: vertical-rl; transform: rotate(180deg);">Widening of K.B.<br/>Proliferative<br/>(Fibrous)<br/>Processes</div> <div style="writing-mode: vertical-rl; transform: rotate(180deg);">Normal<br/>K.B.</div> <div style="writing-mode: vertical-rl; transform: rotate(180deg);">Narrowing of K.B.<br/>Exudative<br/>Processes</div> </div> | NO. | ORIGINAL METHOD<br>% DILUTION OF $\text{CaCl}_2$ | MICROMETHOD<br>0.5% $\text{CaCl}_2$ | <div style="display: flex; flex-direction: column; align-items: center;"> <div style="writing-mode: vertical-rl; transform: rotate(180deg);">Shift to right</div> <div style="writing-mode: vertical-rl; transform: rotate(180deg);">Normal<br/>Zone</div> <div style="writing-mode: vertical-rl; transform: rotate(180deg);">Shift to left</div> </div> |
|--|-----|--|-------------------------------------|--|
|  | 10  | 0.01   | 2 drops                             |  |
|  | 9   | 0.02   | 4 drops                             |  |
|  | 8   | 0.03   | 6 drops                             |  |
|  | 7   | 0.04   | 8 drops                             |  |
|  | 6   | 0.05   | 10 drops                            |  |
|  | 5   | 0.06   | 12 drops                            |  |
|  | 4   | 0.07   | 14 drops                            |  |
|  | 3   | 0.08   | 16 drops                            |  |
|  | 2   | 0.08   | 18 drops                            |  |
|  | 1   | 0.1  | 20 drops                            |  |

In cases of fibrous or proliferative processes, as cirrhosis of the liver, fibrous type of lung tuberculosis, and chronic pneumonia, there is a widening of the K.B. (shift to the right) i.e., flocculation may be present at the eighth tube or higher.

Dees<sup>11</sup> found that this reaction is of assistance in the differential diagnosis between uncomplicated and gangrenous appendicitis. In the uncomplicated case, the K.B. is slightly less than normal, but in the gangrenous condition, the narrowing is marked.

From our experience it is our opinion that the importance of the Weltmann reaction is in the tracing of the course and complications of the disease. For this purpose frequent repetitions of this test during the course of the disease must be carried out, and these require frequent venipunctures.

Havas<sup>12</sup> tried to introduce a micromethod, but his technique was too complicated.

This publication presents a micromethod for the simplification of the Weltmann reaction.

#### TECHNIQUE

The necessary equipment consists of:

- (1) Microcentrifuge tubes of 1 c.c. capacity.
- (2) Special micropipettes, i.e., a blood collecting pipette as used for the micromethod.

- (3) A 0.1 c.c. graduated pipette. The necessary reagent is a 0.5 per cent calcium chloride solution.

Five-tenths cubic centimeter of blood, i.e., 10 drops, is drawn with the blood pipette, placed in the microcentrifuge tube, and centrifuged for fifteen minutes. One-tenth cubic centimeter of the nonhemolyzed serum is pipetted from this tube into a clean Wassermann tube. To it are added 4.9 c.c. of distilled water and 2 drops of the 0.5 per cent calcium chloride solution.

There is now the same concentration as will be found in the tenth tube in the original method. (See table for comparison of the concentrations of the original method and the micromethod.) The contents of the tube are well mixed and boiled over an open flame for one to one and one-half minutes. During this time the tube is continually shaken and observed for flocculation.

If several sera are to be examined, several tubes with the various sera and the necessary solution can be prepared. These tubes are then placed in a boiling water bath for five minutes and observed for flocculation, using either method. If no coagulation is present, 2 more drops of 0.5 per cent calcium chloride are added and the contents are boiled.

In a normal case flocculation occurs after the addition of 8 to 10 drops of the 0.5 per cent solution of calcium chloride, which compares to the sixth or seventh tube in the original method, that is, a concentration of 0.04 to 0.05 per cent.

In comparing the results of the two methods, we found that they check throughout.

The advantages of the micromethod are: (1) less blood is needed, and thus the necessary amount of serum can be obtained by just a puncture of the finger or ear; (2) venipuncture is not necessary, and for this reason daily repetition of the test is possible without much discomfort to the patient; (3) less time is expended in carrying out this method.

#### SUMMARY

A micromethod for the Weltmann reaction is reported. The importance of the test for the diagnosis, course, and prognosis of the different diseases are here stressed. The advantages of this micromethod are mentioned.

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## CONCERNING THE CHOICE OF COMPLEMENT-ANTIGEN COMBINATION FOR USE IN THE KOLMER COMPLEMENT FIXATION TEST\*

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### I. A PRETESTING METHOD FOR COMPLEMENT SELECTION

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AD HARRIS, STATEN ISLAND, N. Y.

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ALMOST as important as the method of preparing antigen is the selection of satisfactory guinea pig serum to be used as complement in the Kolmer complement fixation test for syphilis. Apparently the optimal quality of complement may vary with each individual guinea pig and also may vary from time to time. A complement that is satisfactory with one lot of Kolmer antigen may give unsatisfactory results when used with another Kolmer antigen. Certain complement-antigen combinations which are unsatisfactory may be detected and eliminated by a preliminary control method for testing the combination of reagents.

As reported by Giordano and Carlson,<sup>1</sup> and Kolmer,<sup>2</sup> some guinea pig sera possess a component that is capable of fixing complement in the presence of Kolmer antigen at 6° to 10° C. That some complement sera may also give atypical, zonal false positive reactions with negative human serum has also been reported.<sup>2</sup>

Since this nonspecific reaction of antigen and complement may not occur at 37° C. (during the complement titration), a clue to the presence of this undesirable characteristic of some guinea pig sera is not always available until after the overnight incubation period of the test. The antigen control tube can be expected to show partial or complete inhibition of hemolysis after one hour of secondary incubation at 37° C., when complement that is readily deviated by antigen alone is employed. Under such test conditions the spinal fluid tests may be falsely positive, and some quantitative serum tests may be negative in 0.2 c.c. amounts with positive reactions in the tubes containing smaller amounts of serum. The elimination, from a herd, of guinea pigs whose sera give this type of reaction has not always solved this problem, since previously satisfactory guinea pigs have been found to yield serum having this undesirable property

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at a later date. Nor does the pooling of sera from many guinea pigs yield a complement of usable reactivity in all instances.

The results of the following studies have guided the formulation of a pre-testing procedure which will enable a laboratory, using either lyophilized or fresh complement, to select a satisfactory complement for use with the antigen employed in its Kolmer test.

#### TECHNIQUE

The Kolmer complement fixation test procedure and the test reagents used are those described in Venereal Disease Information Supplement No. 11, 1940. The guinea pigs used were healthy, male animals on a mixed diet of oats, hay, cabbage, and carrots.

#### PROCEDURE

The blood samples were obtained by cardiac puncture from each of 20 guinea pigs, the second and third blood samples were obtained fourteen and twenty-nine days after the initial bleeding. Sera from these bloods were tested with and without antigen to determine the degree of variability in nonspecific complement destruction that might be encountered. Two four-tube sets, containing 1.0 c.c. dilutions of guinea pig serum at 1:30, 1:33, 1:37, and 1:43, were used for each animal tested. To one of these sets of tubes was added a test dose of Kolmer antigen dilution (0.5 c.c.) plus 0.5 c.c. of saline, and to the other set, 1.0 c.c. of saline containing no antigen. All tubes were then placed in the refrigerator at 6° C. for sixteen hours. After a further incubation of ten minutes at 37° C., two units of hemolysin and 0.5 c.c. of cell suspension were added to each tube, and readings were made after a secondary incubation of one hour at 37° C. The results of these testings are listed in Table I.

In Table I the tubes containing antigen are equivalent to the antigen control tubes that would accompany a test at that complement titer, and it is noted that the destruction of complement occurring in these tubes varied within broad limits. Samples of serum, obtained from some guinea pigs on different dates, showed varying degrees of susceptibility to nonspecific destruction of complement. These results point to the fact that some guinea pigs may yield a satisfactory complement at one time and not at another. However, when the three test results are observed, it is evident that all the animals in this group did not show the same degree of serum change nor were all the changes in the same time direction. For this reason, it has been found necessary to test each complement serum at the time of bleeding rather than to rely on any previous test finding.

In order to ascertain the role of antigen in this nonspecific destruction of complement at 6° C., serial dilutions of complement were tested for hemolytic activity after having been in contact with test doses of each of two lots of Kolmer antigen (X and Y) at this temperature for sixteen hours. Control tubes, containing no antigen, were included in this test, as presented in Table II.

The dilutions of complement (1:37, 1:50, and 1:75) used in testing the anti-complementary activities of these two antigens allowed a marked differentiation to be evidenced between them. Since each of these antigens was made under similar conditions, but from different lots of beef heart powder, and since both

TABLE I

RESULTS OF ANTICOMPLEMENTARY EFFECT OF KOLMER ANTIGEN ON THE COMPLEMENT SERA OF 20 GUINEA PIGS BLED AT THREE INTERVALS

| GUINEA<br>PIG | COMPLEMENT DILUTIONS BLED AND TESTED |      |      |      |           |      |      |      |             |      |      |      |
|---------------|--------------------------------------|------|------|------|-----------|------|------|------|-------------|------|------|------|
|               | AUGUST 6                             |      |      |      | AUGUST 20 |      |      |      | SEPTEMBER 4 |      |      |      |
|               | 1:30                                 | 1:33 | 1:37 | 1:43 | 1:30      | 1:33 | 1:37 | 1:43 | 1:30        | 1:33 | 1:37 | 1:43 |
| 1             | 3                                    | 3    | 4    | 4    | 4         | 4    | 4    | 4    | -           | -    | -    | ±    |
| 2             | 4                                    | 4    | 4    | 4    | 4         | 4    | 4    | 4    | 4           | 4    | 4    | 4    |
| 3             | -                                    | -    | -    | ±    | -         | -    | -    | ±    | -           | -    | -    | ±    |
| 4             | -                                    | -    | -    | -    | -         | -    | -    | -    | -           | -    | -    | -    |
| 5             | 2                                    | 3    | 4    | 4    | 3         | 4    | 4    | 4    | -           | -    | ±    | 3    |
| 6             | ±                                    | 1    | 3    | 4    | -         | -    | ±    | ±    | -           | -    | 2    | 3    |
| 7             | 4                                    | 4    | 4    | 4    | 1         | 2    | 3    | 4    | -           | -    | 1    | 3    |
| 8             | ±                                    | 1    | 2    | 3    | -         | -    | -    | -    | -           | -    | -    | -    |
| 9             | 4                                    | 4    | 4    | 4    | 4         | 4    | 4    | 4    | 4           | 4    | 4    | 4    |
| 10            | ±                                    | 1    | 2    | 3    | -         | -    | -    | -    | -           | -    | -    | -    |
| 11            | ±                                    | ±    | ±    | ±    | -         | -    | -    | -    | -           | -    | -    | -    |
| 12            | -                                    | -    | -    | -    | ±         | 1    | 1    | 2    | ±           | ±    | 4    | 4    |
| 13            | -                                    | -    | -    | -    | -         | -    | -    | -    | -           | -    | -    | -    |
| 14            | 3                                    | 3    | 3    | 3    | 1         | ±    | ±    | ±    | -           | -    | -    | -    |
| 15            | -                                    | -    | -    | ±    | -         | -    | -    | -    | -           | -    | -    | -    |
| 16            | -                                    | -    | -    | -    | -         | -    | -    | -    | -           | -    | -    | -    |
| 17            | ±                                    | ±    | 1    | 3    | -         | -    | -    | -    | -           | -    | -    | -    |
| 18            | -                                    | -    | -    | -    | -         | -    | -    | -    | -           | -    | -    | -    |
| 19            | -                                    | -    | -    | -    | -         | -    | -    | -    | -           | -    | -    | -    |
| 20            | -                                    | -    | -    | -    | -         | -    | -    | -    | -           | -    | -    | -    |

Each of the above readings was made on the tubes containing a test dose of Kolmer antigen (0.5 c.c. of 1:640 dilution). Duplicate control tubes of each of the complement dilutions, containing no antigen, gave complete hemolysis in every instance.

These tests were performed with a primary incubation period of sixteen hours at 8° C., plus 10 minutes at 37° C., and readings were made after a secondary incubation period of one hour at 37° C.

The same lot of Kolmer antigen was used throughout these three testings.

TABLE II

RESULTS OF TESTS WITH THE COMPLEMENT SERA OF 30 GUINEA PIGS SHOWING THE ANTICOMPLEMENTARY EFFECT OF TWO LOTS OF KOLMER ANTIGEN

| GUINEA PIG        | COMPLEMENT DILUTION (1.0 C.C.) |      |      |                     |      |      |                  |      |      |
|-------------------|--------------------------------|------|------|---------------------|------|------|------------------|------|------|
|                   | KOLMER ANTIGEN (Y)*            |      |      | KOLMER ANTIGEN (X)* |      |      | NO ANTIGEN ADDED |      |      |
|                   | 1:37                           | 1:50 | 1:75 | 1:37                | 1:50 | 1:75 | 1:37             | 1:50 | 1:75 |
| 1                 | -                              | 2    | 4    | -                   | -    | -    | -                | -    | -    |
| 2                 | 1                              | 3    | 4    | -                   | -    | -    | -                | -    | -    |
| 3                 | -                              | ±    | 3    | -                   | -    | -    | -                | -    | -    |
| 4                 | 3                              | 4    | 4    | -                   | -    | ±    | -                | -    | -    |
| 5                 | ±                              | 4    | 4    | -                   | -    | -    | -                | -    | -    |
| 6                 | ±                              | 1    | 3    | -                   | -    | -    | -                | -    | -    |
| 7                 | -                              | 1    | 4    | -                   | -    | -    | -                | -    | -    |
| 8                 | -                              | -    | 1    | -                   | -    | -    | -                | -    | -    |
| 9                 | -                              | ±    | 1    | -                   | -    | -    | -                | -    | -    |
| 10                | ±                              | 3    | 4    | -                   | -    | -    | -                | -    | -    |
| 11                | -                              | 1    | 4    | -                   | -    | -    | -                | -    | -    |
| 12                | -                              | 4    | 4    | -                   | -    | -    | -                | -    | -    |
| 13                | 4                              | 4    | 4    | -                   | -    | -    | -                | -    | -    |
| 14 to<br>30 incl. | -                              | -    | -    | -                   | -    | -    | -                | -    | -    |

\*Each of these antigens was used in the test dose (0.5 c.c. of 1:640 dilution) amounts.

These tests were performed with a primary incubation period of sixteen hours at 8° C. plus ten minutes at 37° C. Cell suspension (0.5 c.c. of 2 per cent suspension) and 0.5 c.c. of hemolysin, containing 2 units, was then added to all tubes, and final readings were made after a secondary incubation period of one hour at 37° C.

TABLE III

RESULTS OF KOLMER COMPLEMENT FIXATION TESTS PERFORMED ON 20 HUMAN SERA AND 10 SPINAL FLUIDS EMPLOYING TWO LOTS OF KOLMER ANTIGEN AND TWO LOTS OF LYOPHILIZED COMPLEMENT SERA

| NUMBER               | KOLMER<br>ANTIGEN X PLUS<br>COMPLEMENT B | KOLMER<br>ANTIGEN Y PLUS<br>COMPLEMENT B | KOLMER<br>ANTIGEN X PLUS<br>COMPLEMENT A | KOLMER<br>ANTIGEN Y PLUS<br>COMPLEMENT A |
|----------------------|--|--|--|--|
| <i>Sera</i>          |  |  |  |  |
| 1                    | 32±--                                    | 221±-                                    | 132±-                                    | 12311                                    |
| 2                    | -----                                    | -----                                    | -----                                    | -----±3                                  |
| 3                    | -----                                    | -----                                    | -----                                    | -----±13                                 |
| 4                    | -----                                    | -----                                    | -----                                    | -----±21                                 |
| 5                    | -----                                    | -----                                    | -----                                    | -----                                    |
| 6                    | -----                                    | -----                                    | -----                                    | -----±1                                  |
| 7                    | -----                                    | -----                                    | -----                                    | -----±4                                  |
| 8                    | -----                                    | -----                                    | -----                                    | -----±4                                  |
| 9                    | 1±---                                    | 3±---                                    | 12±---                                   | ±±±11                                    |
| 10                   | 11111                                    | 11111                                    | 11111                                    | 11111                                    |
| 11                   | -----                                    | -----                                    | -----                                    | -----±1                                  |
| 12                   | 44±--                                    | 43±--                                    | 112--                                    | 32112                                    |
| 13                   | -----                                    | -----                                    | -----                                    | -----±1                                  |
| 14                   | 12---                                    | 13±--                                    | 13±--                                    | 42112                                    |
| 15                   | -----                                    | -----                                    | -----                                    | -----1                                   |
| 16                   | -----                                    | -----                                    | -----                                    | -----                                    |
| 17                   | ±±---                                    | ±±---                                    | ±±---                                    | -----±1                                  |
| 18                   | -----                                    | -----                                    | -----                                    | -----1                                   |
| 19                   | 44444                                    | 11111                                    | 11111                                    | 11111                                    |
| 20                   | -----                                    | -----                                    | -----                                    | -----                                    |
| <i>Spinal Fluids</i> |  |  |  |  |
| 1                    | ±-----                                   | ±±---                                    | ±±±--                                    | 12344                                    |
| 2                    | 12---                                    | 11---                                    | 112--                                    | 14444                                    |
| 3                    | ±-----                                   | ±-----                                   | 3±---                                    | 14444                                    |
| 4                    | 11---                                    | 1131                                     | 11122                                    | 11111                                    |
| 5                    | ±-----                                   | 1±---                                    | 2±---                                    | 14444                                    |
| 6                    | -----                                    | -----                                    | -----                                    | 44444                                    |
| 7                    | -----                                    | -----                                    | -----                                    | 44444                                    |
| 8                    | -----                                    | -----                                    | -----                                    | 44444                                    |
| 9                    | -----                                    | -----                                    | -----                                    | 44444                                    |
| 10                   | -----                                    | -----                                    | -----                                    | 14444                                    |
| Complement<br>titer  | 1:33                                     | 1:33                                     | 1:33                                     | 1:33                                     |
| Hemolysin titer      | 1:3,000                                  | 1:3,000                                  | 1:3,000                                  | 1:3,000                                  |
| Hemolytic<br>control | Clear 10 min.                            | Clear 10 min.                            | Clear 10 min.                            | Clear 10 min.                            |
| Antigen control      | Clear 15 min.                            | Clear 15 min.                            | Clear 23 min.                            | 4+ after 60 min.                         |

The readings were made ten minutes after the antigen controls cleared in each of the first three groups above. Where Kolmer antigen Y and complement B were used, the tests were read after one hour of secondary incubation.

TABLE IV

A PRETEST METHOD FOR THE SELECTION OF SUITABLE COMPLEMENT FOR THE KOLMER COMPLEMENT FIXATION TEST

| TUBES | 1.0 C.C. OF<br>COMPLEMENT<br>DILUTED<br>1:30 | TEST DOSE<br>OF KOLMER<br>ANTIGEN | KOLMER<br>SALINE | Refrigerator at 0°<br>to 8° C. for 16 to<br>18 hours plus 10<br>min. in 37° C.<br>water bath | 2 UNITS<br>OF<br>HEMOLYSIS | 2% CELL<br>SUSPENSION | Readings made<br>after one hour in<br>37° C. water bath |
|-------|--|-----------------------------------|------------------|--|----------------------------|-----------------------|---|
| 1     | 0.8  | 0.5                               | 0.7              |  | 0.5                        | 0.5                   |   |
| 2     | 0.6  | 0.5                               | 0.9              |  | 0.5                        | 0.5                   |   |
| 3     | 0.4  | 0.5                               | 1.1              |  | 0.5                        | 0.5                   |   |
| 4     | 0.8  | None                              | 1.2              |  | 0.5                        | 0.5                   |   |
| 5     | 0.6  | None                              | 1.4              |  | 0.5                        | 0.5                   |   |
| 6     | 0.4  | None                              | 1.6              |  | 0.5                        | 0.5                   |   |

Mix 0.15 c.c. of guinea pig serum with 4.35 c.c. of Kolmer saline for 1:30 complement dilution.

antigens met with the other test demands and gave maximum fixability at the same dilution (1:640), a comparative evaluation under actual test conditions was arranged. For this purpose, serum from guinea pigs No. 1 to 13 (Table II), inclusive, was pooled and lyophilized, and will be referred to as complement A, and serum from guinea pigs No. 14 to 30 (Table II), inclusive, was pooled and lyophilized, and will be designated as complement B.

A series of 20 blood samples (7 positive and 13 negative) and 10 spinal fluids (5 positive and 5 negative) were tested simultaneously in four separate groups. Each of the antigens (X and Y) was used with each of the complements (A and B), giving the results recorded in Table III.

The tests in which antigen X and complement B were used, as well as those containing antigen Y plus complement B, and antigen X plus complement A, reacted in an orderly manner, i.e., the controls (hemolytic system and antigen) cleared in less than thirty minutes, and the tests were readable in less than one hour of secondary incubation. Antigen Y and complement A proved to be an unsatisfactory combination, since the antigen control tube did not clear, the spinal fluids gave false positive reactions, and many of the serum specimens gave atypical, zonal reactions.

These observations have aided in the formulation of the following preliminary pretest control method for observing the behavior of the complement-antigen combinations. Each guinea pig serum used, whether as fresh complement or as part of a lyophilized lot, is pretested with the antigen with which it will later be used in the Kolmer test. This may be accomplished by bleeding guinea pigs into individual test tubes, allowing these tubes to remain at room temperature until a firm clot is formed, and then placing them in the refrigerator for a few hours. After this period the clots are loosened with a wooden applicator, centrifuged at high speed for a few minutes, and the 0.15 c.c. serum needed for pretesting is removed from each tube. The tubes containing clot and serum are then placed in the refrigerator until the following morning. This testing method is outlined in Table IV.

Guinea pig sera that gave complete hemolysis in all six tubes of this test have been found to be of good quality and to have the degree of hemolytic activity required by the test author (1:30 to 1:43 for two full units). A guinea pig serum showing an equal inhibition of hemolysis in both the antigen tubes, and those containing no antigen, may be considered usable but will usually be found to have a lower hemolytic activity, as determined by the complement titration on the following day. Any sera causing a greater inhibition of hemolysis in the presence of antigen than is found in the tubes containing no antigen, may be considered as unsatisfactory when used with the testing antigen.

Using this pretesting method, more than twenty lots of lyophilized complement, satisfactory for use in the Kolmer complement fixation test, have been prepared. Each of these lots of complement was pooled from 25 to 31 guinea pigs, obtained from random herds from several breeding sources. Fifty lots of fresh complement (5 guinea pigs each), chosen in the same manner, have all been found to be of satisfactory reactivity.

## DISCUSSION

Kolmer has stated that “. . . prezone and nonspecific complement-fixation tests are due to factors involving the complement employed . . .,” and it appears that the degree of this reactivity may also be influenced by some antigen component. Whereas antigen Y with complement A proved to be an unsatisfactory combination, antigen Y with complement B, and complement A with antigen X, gave satisfactory test results. Therefore, antigen Y or complement A can be considered usable if combined with a suitable complement or antigen, respectively, but are entirely unsatisfactory when used together.

When fresh complement is employed, the preliminary antigen-complement combinations of the pretest procedure can be placed in the refrigerator the afternoon the blood is drawn. This can be arranged by removing the small amount of serum needed for testing from each tube and allowing the remainder to stay on the clot until the following morning. After a reading has been made on each serum, sera found to have satisfactory reactivity can be pooled, titrated, and used without further delay. When large quantities of serum are to be lyophilized, a small amount of blood (0.5 c.c. is adequate) may be obtained from each guinea pig and tested the day before exsanguination. However, the routine used for fresh complement can be applied to complement for lyophilization if the whole blood is kept at a low temperature, during the overnight storage period, and the dehydration process is started as soon as possible after the pooling of the satisfactory sera. This pretesting procedure for complement selection is not offered as a substitute for any part of the Kolmer titrations, but rather as an adjunct to the complement titration.

## SUMMARY

1. The suitability of a guinea pig complement for use with a specific Kolmer antigen may vary, within broad limits, in some guinea pigs during fourteen- to twenty-three-day intervals.
2. As demonstrated by a sixteen-hour incubation period at 6° C., the anti-complementary action of two lots of Kolmer antigen, having identical test dose titers, was not similar on all complements tested.
3. A pretest method for complement selection is described.

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## CHEMICAL

### THE CHOLINE CONTENT OF BIOLOGICAL FLUIDS\*

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IN THE course of some studies on the presence and significance of choline in cortico-adrenal extracts<sup>1</sup> a survey was made of the available methods for the quantitative determination of choline in relatively small amounts of fluid. Although the widespread occurrence of choline and its compounds in the normal organism had been regarded as some indication of functional significance,<sup>1</sup> and although choline and its compounds have become increasingly important in recent years because of the unique role of an essential dietary factor as well as a hormone, little or no quantitative data can be found on the choline content of the various biological fluids.

The biological methods for choline determination depend on the conversion of the choline to its much more powerful acetic ester, and the subsequent assay of the latter on some test object by comparison with standard acetylcholine solutions. Chemically, choline forms precipitates with many reagents, but of these only three (the double salts with iodine, platinum chloride, and mercuric chloride) can be formed when the choline is in dilution of 1:2,000,000.

The original method of Sharpe<sup>2</sup> was based on the work of Stanek,<sup>3</sup> who precipitated choline as the unstable periodide and estimated the iodine by titration with sodium thiosulfate. Roman,<sup>4</sup> however, suggested the use of a stronger solution of iodine, precipitating the choline as the enneaiodide ( $C_5H_{14}NOI_9$ ). Many investigators have used some modification of the latter procedure. The objections of this procedure are: (a) It requires at least 10 to 20 c.c. of blood. (b) Extraction by heating on a boiling water bath (presumably to split choline esters) involves the loss of free choline by decomposition, and increases the possibility of lecithin hydrolysis, resulting in newly liberated choline. (c) Dialysis for twenty-four hours against distilled water and reduction in volume of the dialysate offer excellent possibilities for the loss of choline, even up to 100 per cent. (d) Choline periodide and enneaiodide are unstable products and losses occur during evaporation to small volume. (e) The ice-cold water (0 to 1° C.) used to wash away the excess iodine dissolves some of the soluble precipitate. (f) The reagent likewise precipitates ammonium salts, stachydrine, and other extractives which cannot be removed completely without likewise affecting the precipitate.

Precipitation of the choline as the double salt with platinum chloride or Reinecke's acid may be used, and the resulting precipitates may be purified and weighed as such, checked by melting point determinations, analyzed for nitrogen, or converted to some other salt (e.g., gold salt) which can be assayed

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biologically. In any case, large amounts of the original choline-containing fluid would be required for such procedure.

Addition of a saturated solution of mercuric chloride in absolute alcohol to an alcoholic solution of choline results in the formation of a stable precipitate of choline mercury chloride ( $C_5H_{11}ONCl \cdot 6HgCl_2$ ), melting point  $242^\circ$ - $243^\circ$ . The double salt separates out as extremely insoluble needles which can be centrifuged and thoroughly washed without loss. The mercury content can then be determined by volumetric analysis.

#### METHOD

The method used in the present study is essentially that of Ambo and Aoki<sup>3</sup> and, with modifications, is given in detail as follows: Two to 5 c.c. of blood are measured into 10 to 25 c.c. of 95 per cent alcohol containing 2 to 3 drops of acid alcohol, shaken, and allowed to stand for twenty-four hours for precipitation and extraction. The mixture is then filtered, the residue is washed well with a fine stream of 95 per cent alcohol, and the total filtrate is carefully evaporated under reduced pressure to almost dryness. It is important to avoid splashing and to keep the temperature of the bath below  $40^\circ$  C. at all times. The residue (a few drops) is extracted three times with petroleum ether to remove fat and phosphatides, the petroleum ether being removed in each case by centrifuging and drawing off the supernatant fluid with a medicine dropper drawn out to a fine capillary. The remaining small amount of liquid is then evaporated to dryness under reduced pressure, the residue is extracted three times with small amounts of cold absolute alcohol and filtered through a very small funnel into a 50 c.c. flask (thus removing the material insoluble in cold alcohol). The filtrate is then brought to a very small volume (0.1 to 0.2 c.c.) under reduced pressure, and 1 to 2 c.c. of a saturated solution of mercuric chloride in absolute alcohol is then added. The flask is shaken well and placed in the refrigerator for twenty-four hours for complete precipitation of the choline mercury chloride.

The contents of the flasks (the precipitate and the excess mercuric chloride in absolute alcohol) are then transferred to centrifuge tubes and centrifuged rapidly at 2,800 r.p.m., and the clear, supernatant fluid is removed with a capillary medicine dropper and discarded. Absolute ether is used to wash and transfer the contents of the flask to the centrifuge tube. The precipitate is then washed six times with absolute ether, with centrifuging and removal of the supernatant fluid after each washing. It is essential that the precipitate and its container be washed completely free from excess mercuric chloride. The precipitate is then dissolved in 1 c.c. of 10 per cent potassium iodide and transferred to a flask, to which are then added 1 c.c. of 15 per cent potassium hydroxide, 3 drops of mucilage of acacia (U.S.P.) and, while shaking, 1 c.c. of 11.5 per cent formaldehyde. The free mercury separates out in fine suspension, which is first shaken well and then allowed to stand for five minutes. The mixture is then acidified with 6 c.c. of 20 per cent glacial acetic acid and 2 c.c. of 0.005 N potassium iodate solution are added from a microburette. After shaking gently to enable solution of mercury (the freed iodine combined with mercury), the excess potassium iodate is titrated with 0.005 N sodium thiosulfate, with



starch as an indicator. The 0.005 normal solutions of potassium iodate and sodium thiosulfate are freshly prepared from standardized 0.1 N stock solutions; the former are standardized at the time of titration of the regularly prepared samples.

*Calculation:* Each 0.1 c.c. of 0.005 N potassium iodate used combines with 0.067 mg. mercuric chloride, which is equivalent to 0.0058 mg. choline chloride containing 0.005 mg. choline.

There are several advantages of this method: (1) A small amount of the original fluid is used, so that two or more analyses may be performed on the same sample. (2) The disadvantages of choline instability in aqueous solution, and its volatility on reduction to small volume on a water bath, are avoided by the use of absolute alcohol as a solvent and distillation under reduced pressure for concentration to small volume. (3) The free choline is separated from interfering material without loss and without the possibility of liberation of bound choline from lecithin, sphingomyelin, etc. (4) The precipitant is effective even in a 1:2,000,000 dilution of choline and forms a stable, insoluble precipitate. (5) The mercury content of the precipitate may be accurately determined by standard quantitative inorganic procedure, from which the choline content is readily calculated.

### RESULTS

The recovery of choline from standard solutions of choline chloride in 95 per cent alcohol is shown in Table I. The range of recovery is from 87.6 to 118.6 per cent; the average recovery for the series is 99.5 per cent.

TABLE I  
RECOVERY OF CHOLINE FROM STANDARD ALCOHOLIC SOLUTIONS

| CHOLINE PRESENT<br>(GAMMA) | CHOLINE RECOVERED |            |
|----------------------------|-------------------|------------|
|                            | (GAMMA)           | (PER CENT) |
| 14.3                       | 13.5              | 94.4       |
| 29.0                       | 27.0              | 93.2       |
| 29.0                       | 26.0              | 89.7       |
| 35.9                       | 37.5              | 104.3      |
| 33.0                       | 37.0              | 112.0      |
| 33.0                       | 35.0              | 106.0      |
| 86.0                       | 76.0              | 88.4       |
| 104.0                      | 102.0             | 98.1       |
| 113.0                      | 99.0              | 87.6       |
| 120.0                      | 106.0             | 88.4       |
| 125.0                      | 120.0             | 96.0       |
| 136.0                      | 140.0             | 102.8      |
| 274.0                      | 245.0             | 89.4       |
| 431.0                      | 407.0             | 94.5       |
| 431.0                      | 467.0             | 108.3      |
| 666.0                      | 790.0             | 118.6      |
| 666.0                      | 754.0             | 113.2      |
| 666.0                      | 701.0             | 105.2      |
| 856.0                      | 842.0             | 98.3       |
| 862.0                      | 870.0             | 100.9      |
| 862.0                      | 864.0             | 100.2      |

Two standard choline chloride solutions (containing 6.66 and 19.83 mg. of choline per cubic centimeter) were submitted for nitrogen analysis in the chemistry laboratory of Billings Hospital. On the basis of the reported nitrogen content, these solutions had choline equivalents of 6.52 and 19.45 mg. per cubic

centimeter, 97.9 and 98.1 per cent, respectively, of the calculated choline content. These solutions were subsequently used to determine the yield of choline mercury chloride from solutions of known choline content (average yield 98.4 per cent), and the per cent of choline recovery from known amounts of choline mercury chloride dissolved in 10 per cent potassium iodide solution. In a series of 11 control experiments made on four such potassium iodide solutions (whose choline equivalents were 1.208, 0.683, 0.418, and 0.280 mg. per cubic centimeter) the range of choline recovery was from 88.3 to 102.3 per cent; the average recovery for the series was 93.4 per cent.

TABLE II  
RECOVERY OF CHOLINE ADDED TO BIOLOGICAL FLUIDS

| ORIGINAL FLUID                | CHOLINE<br>CONTENT<br>( $\gamma$ MM) | CHOLINE<br>ADDED<br>( $\gamma$ MM) | TOTAL<br>CHOLINE<br>PRESENT<br>( $\gamma$ MM) | TOTAL CHOLINE<br>RECOVERED |            |
|-------------------------------|--------------------------------------|------------------------------------|---|----------------------------|------------|
|                               |                                      |                                    |   | ( $\gamma$ MM)             | (PER CENT) |
| 1 c.c. serum ♂                | 11.6                                 | 29.6*                              | 41.2  | 37.8                       | 91.7       |
| 2 c.c. blood ♂                | 25.6                                 | 43.0                               | 68.6  | 61.2                       | 89.1       |
| 2 c.c. blood ♂                | 37.4                                 | 43.0                               | 80.4  | 70.7                       | 87.9       |
| 2 c.c. blood ♀                | 28.4                                 | 86.0                               | 114.1   | 101.8                      | 89.0       |
| 2 c.c. blood ♀                | 30.1                                 | 86.0                               | 116.1   | 106.0                      | 91.3       |
| 3 c.c. blood ♀                | 37.8                                 | 86.0                               | 123.8   | 112.2                      | 90.6       |
| 1 c.c. menstrual blood        | 32.2                                 | 215.0                              | 247.2   | 211.3                      | 85.5       |
| 2 c.c. cow's milk             | 162.0                                | 323.8                              | 485.8   | 425.9                      | 87.7       |
| 1 c.c. human semen            | 337.0                                | 323.8                              | 660.8   | 604.6                      | 91.5       |
| 1 c.c. corticoadrenal extract | 1324.0                               | 772.5                              | 2096.5  | 2120.0                     | 101.1      |

\*1 c.c. of a 1:10 dilution of human semen.

Table II gives the results obtained in control experiments in which known amounts of standard alcoholic choline chloride solutions were added to various biological fluids. The range of total choline recovery was from 85.5 to 101.1 per cent; the average recovery for the series was 90.5 per cent.

TABLE III  
CHOLINE CONTENT OF BIOLOGICAL FLUIDS

| NO. | MATERIAL                | NO.<br>SAMPLES | HIGHEST<br>VALUE<br>(MG./LITER) | LOWEST<br>VALUE<br>(MG./LITER) | AVERAGE<br>VALUE<br>(MG./LITER) |
|-----|-------------------------|----------------|---------------------------------|--------------------------------|---------------------------------|
| 1   | Human saliva ♂ (a)      | 12             | 14.4                            | 8.6                            | 11.6                            |
| 2   | Human saliva ♂ (b)      | 7              | 9.9                             | 4.7                            | 6.5                             |
| 3   | 1 and 2 combined        | 19             | 14.4                            | 4.7                            | 9.7                             |
| 4   | Human saliva ♀ (a)      | 75             | 36.4                            | 6.2                            | 16.9                            |
| 5   | Human blood ♂ (c)       | 8              | 17.1                            | 10.2                           | 13.2                            |
| 6   | Human blood ♂ (d)       | 10             | 22.8                            | 8.5                            | 13.5                            |
| 7   | 5 and 6 combined        | 18             | 22.8                            | 8.5                            | 13.4                            |
| 8   | Human blood ♀ (d)       | 19             | 21.8                            | 9.4                            | 14.6                            |
| 9   | Human blood, pregnant ♀ | 62             | 19.9                            | 9.9                            | 13.1                            |
| 10  | Human semen             | 17             | 477.0                           | 234.0                          | 341.0                           |
| 11  | Dog bladder bile        | 7              | 380.0                           | 78.0                           | 250.0                           |
| 12  | Dog lymph               | 3              | 23.5                            | 14.2                           | 18.2                            |

(a) Patients chewed paraffin and collected samples in bottles.

(b) Dilute saliva collected by continuous use of dental siphon.

(c) Normal persons.

(d) Patients from Out-Patient Department of Billings Hospital.

The results of choline analyses on most of the biological fluids studied are summarized in Table III. All analyses were done in duplicate; in many cases they were repeatedly performed on the same sample to determine the degree of

accuracy of triplicate and quadruplicate analyses on the same sample through a wide range of choline concentrations. The total number of analyses, including those on standard solutions and miscellaneous samples, exceeded 650. Detailed studies on blood choline variations in pregnancy and salivary choline variations during the menstrual cycle will be presented in subsequent papers.

#### SUMMARY

1. A modified chemical method for the quantitative determination of choline in small amounts of biological fluids has been described in detail.
2. Control studies have been made to show choline recovery from standard alcoholic choline chloride solutions, from solutions of known choline mercury chloride content, and from biological fluids to which known amounts of choline were added.
3. The choline content of 220 samples of various biological fluids is reported.

Grateful acknowledgment is made to Dr. A. J. Carlson for his cooperation throughout the course of this work.

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## EFFECT OF ADDED SALTS ON RECOVERY OF HIPPURIC ACID FROM URINE\*

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IT HAS generally been found that hippuric acid varies in solubility in different urines, the loss on recovery being too variable for reasonably accurate clinical results of the Quick liver function test as originally outlined.<sup>1, 2</sup> Efforts have been made to reduce this loss, at least to a constant value. Several years ago Berg and Mattill<sup>3</sup> recommended the use of ammonium sulfate in student laboratory procedure for the isolation of hippuric acid from urine. A modification of their procedure has been used in this laboratory in liver function tests for three years with excellent results. Quick revised his analytical method to include the use of ammonium sulfate,<sup>4</sup> but has not published the experimental work.<sup>5</sup> Sodium chloride<sup>6</sup> has also been employed to reduce the hippuric acid solubility in urine, but the results are not directly applicable to the conditions of the liver function test where recovery losses need to be known for a urine solution of a salt of the hippuric acid.

This paper presents a study of the effect of the addition of ammonium sulfate and some other salts on the loss in recovery of hippuric acid from urine in gravimetric procedure. Volumetric analysis is less desirable, since added salts affect the titration values, and it has been found far less precise in the hands of a number of technicians.

### EXPERIMENTAL

Fresh, clear urine specimens were picked at random from routine hospital specimens in order to have representative results. Those containing more than a trace of albumin, or having unusual color, were rejected. To simulate the chemical composition of urine as it is passed in Quick liver function tests, hippuric acid (0.80 Gm.) was added as a solution of the sodium salt, prepared by neutralizing the acid with dilute sodium hydroxide. The desired amount of a salt to be tested for its effect in reducing the solubility of hippuric acid was then dissolved in the urine, and the mixture was run through a paper of rapid filtering quality, or centrifuged to remove the solids which separated. A solid phase which contained no hippurate separated from all urines when sufficient amounts of a salt were added. Therefore, the minimum quantity of a salt to be used was the amount necessary to condition hippurate-free urine to the point where no further separation of solids occurred upon acidification.

After filtration 1 c.c. of concentrated sulfuric acid was added for each 100 c.c. of urine used. Added sodium hippurate solution was considered part of the urine volume. The solution was stirred until several minutes after hippuric acid precipitated. In order to achieve constant results it was necessary to start

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crystallization by friction between the beaker and stirring rod. One to four hours later the precipitate was collected quantitatively on a weighed filter paper over suction. The filtrate was used to wash the beaker. The precipitate and the inside of the funnel were washed with 15 c.c. of distilled water (25° C.) from a medicine dropper. This left less than 0.01 Gm. of the precipitating agent on the filter and had a negligible effect on the quantity of hippuric acid. Weight of the precipitate was taken after it had dried twelve hours at 37° C. A 5.5 cm. No. 4 Whatman filter on a 67 mm. Buchner porcelain funnel fitted into a side-neck test tube was found convenient, because all the solid settled on the paper, and any loss could be seen in the tube and recovered.

## DISCUSSION AND RESULTS

By this method the true solubility of hippuric acid in treated urine was not determined, but the loss incurred in actual procedure was ascertained. The variations in recovery from different specimens is probably largely due to the inability of added salts to abate entirely the effects of urinary colloids on crystallization.

TABLE I

LOSS OF HIPPURIC ACID IN URINES TO WHICH SALTS HAVE BEEN ADDED TO FACILITATE PRECIPITATION

| SPECIMEN                | URINE VOLUME<br>(C.C.) | HIPPURIC ACID<br>LOSS<br>(GM./100 C.C.) | SPECIMEN                 | URINE VOLUME<br>(C.C.) | HIPPURIC ACID<br>LOSS<br>(GM./100 C.C.) |
|-------------------------|------------------------|---|--------------------------|------------------------|---|
| <i>Ammonium Sulfate</i> |                        |   | <i>Magnesium Sulfate</i> |                        |   |
| 1                       | 75                     | 0.116                                   | 22                       | 75                     | 0.140                                   |
| 2                       | 75                     | 0.120                                   | 23                       | 75                     | 0.180                                   |
| 3                       | 75                     | 0.080                                   | 24                       | 75                     | 0.152                                   |
| 4                       | 75                     | 0.124                                   | 25                       | 75                     | 0.168                                   |
| 5                       | 75                     | 0.146                                   | 26                       | 75                     | 0.140                                   |
| 6                       | 75                     | 0.140                                   | 27                       | 75                     | 0.121                                   |
| 7                       | 75                     | 0.125                                   | 28                       | 75                     | 0.149                                   |
| 8                       | 75                     | 0.066                                   | 29                       | 75                     | 0.152                                   |
| 9                       | 75                     | 0.105                                   | 30                       | 100                    | 0.052                                   |
| 10                      | 75                     | 0.140                                   | 31                       | 100                    | 0.076                                   |
| 11                      | 75                     | 0.144                                   | 32                       | 200                    | 0.128                                   |
| 12                      | 100                    | 0.085                                   | 33                       | 200                    | 0.139                                   |
| 13                      | 100                    | 0.067                                   | 34                       | 200                    | 0.056                                   |
| 14                      | 100                    | 0.067                                   | 35                       | 200                    | 0.121                                   |
| 15                      | 150                    | 0.078                                   | Average                  |                        | 0.127                                   |
| 16                      | 200                    | 0.091                                   | <i>Sodium Sulfate</i>    |                        |   |
| 17                      | 200                    | 0.090                                   | 36                       | 75                     | 0.170                                   |
| 18                      | 200                    | 0.036                                   | 37                       | 75                     | 0.166                                   |
| 19                      | 200                    | 0.105                                   | 38                       | 75                     | 0.157                                   |
| 20                      | 200                    | 0.056                                   | 39                       | 75                     | 0.139                                   |
| 21                      | 200                    | 0.113                                   | Average                  |                        | 0.158                                   |
| Average                 |                        | 0.100                                   |                          |                        |   |

Table I gives the hippuric acid loss for 21 different urine specimens to which 35 Gm. of ammonium sulfate were added for each 100 c.c. volume. This is the minimum quantity of that salt practical for use; more of it has no advantage. For clinical accuracy 0.1 Gm. can be added to the amount of hippuric acid recovered from each 100 c.c. of urine by the method outlined here. This holds true for urine volumes to 200 c.c. without concentration. However, the necessity of concentrating urine from patients with copious output is not obviated.

Magnesium sulfate is a more economical salt to add to urine. Experimental results with the use of this salt show slightly greater average loss per 100 c.c.; nevertheless, the data in Table I indicate it is a good agent to use. Fifty grams per 100 c.c. of urine are necessary. Magnesium sulfate in this concentration is still far enough from the saturation point that it does not readily crystallize out. An average loss of 0.127 Gm. per 100 c.c. of urine is to be added to the hippuric acid recovery if magnesium sulfate is employed at 50 Gm. per 100 c.c.

Four determinations with 35 Gm. of sodium sulfate per 100 c.c. of urine gave an average loss of 0.158 Gm. of hippuric acid per 100 c.c. Addition of more of this salt was found impractical.

Thirty grams of sodium chloride per 100 c.c. of urine reduced the loss to 0.1 Gm. or less. Since it is more difficult than the other salts used to wash from the hippuric acid, it probably contributed to the recovery in the trials made. Moreover, the effective concentration of salt is so close to its saturation point in urine that difficulty is experienced with its crystallizing from solution and on the filter. Its use in either volumetric or gravimetric method for hippuric acid lengthens the procedure because heat is necessary to effect solution, and this must be followed by a cooling period.

#### SUMMARY

Of four salts tested for reducing the solubility of hippuric acid in urine to a constant value, ammonium sulfate was found to be the best. The loss was reduced to an average of 0.1 Gm. per 100 c.c. of urine under the conditions of the Quick liver function test, using gravimetric procedure.

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## A NOTE ON THE USE OF TÖPFER'S REAGENT IN GASTRIC ANALYSIS AFTER TEST MEALS OF MILK, CREAM, OR SOYBEAN BISCUITS\*

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IN TESTING a patient with duodenal ulcer with various foods to see whether any of them would decrease the gastric acidity, it was found that milk would give about one-half as much free acid as the bread test meal, using Töpfer's reagent as the indicator. Soybean biscuits also decreased the acidity. Casein and whey were then used, but neither of these showed any apparent change in the total free acid. Finally, sweet cream was fed after which no free acid was indicated by Töpfer's in the fractional test meal. Likewise, Töpfer's failed to indicate change of acidity when an excess of hydrochloric acid was added to cream *in vitro*.<sup>1</sup> Since the salts of milk should give it more buffering action than cream, the problem was investigated further.

Preliminary experiments on the titration of milk and cream with hydrochloric acid, using Töpfer's reagent as the indicator, suggested that the indicator did not function properly in the presence of these substances. To check the action of the indicator, potentiometric titrations were carried out using a Leeds and Northrup pH meter with glass electrode. Stepwise additions of N/10 hydrochloric acid and sodium hydroxide produced the expected changes in pH. With phenolphthalein as the indicator sodium hydroxide caused the solution to turn pink at pH 8.5. However, with Töpfer's reagent as the indicator the solution remained yellow even after the acidity was lowered to pH 1.5. Further addition of 2 ml. of concentrated sulfuric acid caused the solution to turn red, which should have occurred at pH 2.9.

A sample of milk was acidified with N/10 hydrochloric acid to pH 2.5. Töpfer's reagent was added, and the indicator was still yellow. After centrifuging it was noted that the fat and protein layer had a yellow color, while the milk serum was slightly pink. On adding one more drop of Töpfer's reagent to the milk serum it turned red.

These results indicate that the fat and/or proteins of the milk interfere with the use of Töpfer's reagent as an indicator in the titration of milk with hydrochloric acid. It was now thought desirable to determine whether the fat or the protein was responsible for this interference.

(a) Fresh skim milk was obtained which contained less than 0.02 per cent fat. On titration with hydrochloric acid, Töpfer's reagent functioned normally and changed at the proper pH. Thus it appears as though the casein is not the interfering factor.

(b) A sample of 20 per cent cream was acidified in the presence of an excess of Töpfer's reagent and centrifuged. The serum was pink, but the fat-

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protein layer had a yellow color. The casein was then removed by washing three times with N/10 sodium hydroxide, once with water, twice with N/10 hydrochloric acid, and once again with water. After this treatment the fat layer still retained the yellow color.

It appears probable that in this two-phase system the ionization of the Töpfer's reagent in the fat phase is negligible.

#### TITRATION OF SOYBEAN MEAL

Ten grams of soybean meal were suspended in 90 ml. of distilled water and titrated with hydrochloric acid in the presence of Töpfer's reagent.

During the titration with this acid, Töpfer's reagent remained yellow even after the pH was as low as 2.1 as determined potentiometrically. Again on the addition of 1 ml. of concentrated sulfuric acid, the reagent turned red. In this case, however, it was noted that if during the titration, 1 drop of the solution was removed and tested with Töpfer's reagent on a spot plate, the indicator turned red at the proper pH. This again indicated that some constituent of the soybean meal interferes with the use of Töpfer's reagent.

The pH range over which Töpfer's reagent changes color is wide, and thus even at best the use of the reagent is inaccurate. This fact, coupled with the interfering substances in milk, cream, soybean flour, and possibly other common foodstuffs, makes the use of Töpfer's reagent inadvisable in gastric analyses involving these foods. However, in routine gastric analysis, where the meal of bread and water is fed, it was found that Töpfer's reagent was satisfactory and agreed with potentiometric titration.

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## A NOTE ON THE DECOMPOSITION OF NESSLER'S SOLUTION\*

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RECENTLY we noticed that freshly prepared Nessler's solution on standing a short time in rubber-stoppered bottles formed a yellow precipitate. New solutions of Nessler's stock reagent and 10 per cent sodium hydroxide were prepared and mixed, and again cloudiness and precipitation occurred.

Another set of reagent bottles was obtained, and cork stoppers were used in place of rubber. No precipitation was observed with either the old or the new solutions. When rubber stoppers were substituted for cork, precipitation was noted. While trying to locate the cause of our trouble, we remembered that we had removed the bloom from rubber stoppers with acetone according to the method of Matviak.<sup>1</sup> The stoppers were not immersed in acetone but were wiped with a cloth moistened with acetone. They were washed thoroughly in water, dried and exposed to room air for a day or two prior to their use. No odor of acetone was detected from these stoppers.

The addition of a drop of acetone to Nessler's reagent caused immediate precipitation, and a like result was obtained when acetone vapor was blown over the mouth of a test tube containing Nessler's solution. Fumes arising from the use of acetone in the laboratory produced cloudiness, showing the extreme sensitivity of Nessler's reagent to acetone.

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Received for publication, August 7, 1941.

## DETERMINATION OF CALCIUM IN BILE\*

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THE wet ashing method with nitric acid and perchloric acid, as described for grains by Giesiking, Snider, and Getz,<sup>1</sup> and for tissues by Buell,<sup>2</sup> may be applied to bile. It gives a water-soluble ash which is very satisfactory for the determination of calcium.

The procedure is as follows: Add to from 1.0 to 3.0 c.c. of hepatic bile in a 50 c.c. beaker twice the quantity of nitric acid and an equal amount of perchloric acid. For gall bladder bile use 0.5 to 1.0 c.c. samples and add four times the amount of nitric acid and twice the amount of perchloric acid. Heat to dryness at low heat on the electric hot plate. Take up the residue in water

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and transfer quantitatively to a 15 c.c. centrifuge tube, keeping the volume below 6 c.c. Add a drop of methyl red, enough ammonia to make the solution alkaline, and then 10 per cent acetic acid to approximately pH 4 to 5. Add 1 c.c. of ammonium oxalate (4 per cent) and proceed as in the Clark-Collip method for determination of calcium in blood.

Table I gives the results of some analyses.

TABLE I

| BILE                | AMOUNT<br>TAKEN<br>(c.c.)                     | POTASSIUM<br>PERMANGANATE<br>(c.c. 0.01N) | CALCIUM<br>(MG./<br>SAMPLE) | CALCIUM<br>FOUND<br>(MG./<br>100 c.c.) | CALCIUM<br>THEORETICAL<br>(MG./<br>100 c.c.) | RECOVERY<br>(%) |
|---------------------|---|---|-----------------------------|--|--|-----------------|
| Human hepatic       | 2.0   | 0.40<br>0.38<br>0.36<br>0.37              | 0.076                       | 3.8                                    | Avg. 3.8                                     |                 |
|                     | 1.0 +<br>1 mg. Ca<br>(1.0 c.c.<br>solution)*  | 0.38<br>5.02<br>5.04<br>5.07<br>5.08      |                             |  |  |                 |
|                     |   | 5.05                                      |                             |  |  |                 |
|                     |   |   | 1.010                       | 50.6                                   | 51.9   | 97.5            |
|                     |   |   |                             |  |  |                 |
|                     |   |   |                             |  |  |                 |
| Dog hepatic         | 1.0   | 0.88<br>0.80                              | 0.170                       | 17.0                                   | Avg. 16.4                                    |                 |
|                     | 2.0   | 0.85<br>1.60<br>1.64                      |                             |  |  |                 |
|                     | 3.0   | 2.41<br>2.41                              | 0.324                       | 16.2                                   |  |                 |
|                     | 3.0 +<br>0.5 mg.<br>Ca                        | 4.06<br>4.79                              | 0.482                       | 16.0                                   |  |                 |
|                     | 1.0 +<br>1.0 mg.<br>Ca                        | 4.72<br>5.69                              | 0.944                       | 27.0                                   | 28.0   | 96.4            |
|                     |   |   | 1.138                       | 56.9                                   | 58.2   | 97.7            |
| Dog gall<br>bladder | 0.5   | 0.90<br>0.91                              | 0.182                       | 36.4                                   |  |                 |
|                     | 1.0   | 0.91<br>1.77                              |                             |  |  |                 |
|                     |   | 1.84                                      | 1.80                        | 0.360                                  | 36.0   |                 |
| Dog hepatic         | 1.0   | 0.61<br>0.60                              | 0.122                       | 12.2                                   | Avg. 12.4                                    |                 |
|                     | 2.0   | 0.61<br>1.20<br>1.21                      |                             |  |  |                 |
|                     | 3.0   | 1.21<br>1.84                              | 0.242                       | 12.1                                   |  |                 |
|                     | 5 c.c. + 0.5<br>mg. Ca                        | 1.87<br>0.97                              | 0.372                       | 12.6                                   |  |                 |
|                     | 5 c.c. + 1.0<br>mg. Ca                        | 1.01<br>0.99                              | 0.198                       | 19.8                                   |  | 97.5            |
|                     | 5 c.c. + 1.0<br>mg. Ca                        | 0.66<br>0.66                              | 0.132                       | 26.4                                   | 27.0   | 97.9            |
|                     | 5 c.c. + 1.0<br>mg. Ca                        | 1.24<br>1.25                              | 0.250                       | 25.0                                   | 27.0   | 92.6            |
|                     |   | 1.25                                      |                             |  |  |                 |
|                     |   |   |                             |  |  |                 |
|                     |   |   |                             |  |  |                 |
| Human hepatic       | 1.0   | 0.30<br>0.31                              | 0.062                       | 6.2                                    | Avg. 6.1                                     |                 |
|                     | 2.0   | 0.31<br>0.60<br>0.57                      |                             |  |  |                 |
|                     | 3.0   | 0.59<br>0.92<br>0.96                      | 0.118                       | 5.9                                    |  |                 |
|                     | 5 c.c. + 0.5<br>mg. Ca (0.5<br>c.c. solution) | 0.77<br>0.78                              | 0.188                       | 6.2                                    |  |                 |
|                     | 5 c.c. + 1.0<br>mg. Ca (1.0<br>c.c. solution) | 0.78<br>0.78                              | 0.156                       | 15.6                                   | 14.7   | 106.1           |
|                     | 5 c.c. + 1.0<br>mg. Ca (1.0<br>c.c. solution) | 0.58<br>0.60                              | 0.118                       | 23.6                                   | 21.8   | 108.2           |
|                     | 5 c.c. + 1.0<br>mg. Ca (1.0<br>c.c. solution) | 0.59<br>1.11<br>1.10                      | 0.222                       | 22.2                                   | 21.8   | 101.8           |
|                     |   | 1.11                                      |                             |  |  |                 |
|                     |   |   |                             |  |  |                 |
|                     |   |   |                             |  |  |                 |

\*A solution containing 1 mg. of calcium per cubic centimeter was used in all instances where calcium was added to the bile.

Usually the residue obtained is white in color and dissolves readily in water. Occasionally a brown residue is obtained, which may be digested to whiteness by adding nitric acid and perchloric acid again. This is not necessary, however, as good results are obtainable even when the residue is slightly discolored. We have also found that if large amounts of organic material are present, as with gall bladder bile, the digestion mixture may turn dark brown or black when it reaches a small volume. If this happens, a second, or even a third, digestion with nitric acid and perchloric acid is necessary. In such instances care should be taken not to allow the dark mixture to evaporate to dryness since slight explosions may occur.

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### FURTHER NOTES ON THE COLORIMETRIC DETERMINATION OF INULIN IN BLOOD AND URINE\*

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WITH the development of a practical clinical method for measuring the glomerular filtration rate in man, clinical laboratories have been called upon to determine the concentration of inulin in blood and urine.<sup>1</sup> For this purpose a simple direct colorimetric method was described by Alving, Rubin, and Miller.<sup>2</sup> Certain difficulties which have been experienced by some laboratories in the routine application of this method may be overcome by the use of the following modifications and precautions.‡

**Reagents.**—Diphenylamine reagent. This reagent is now prepared as follows: To 250 c.c. of absolute ethyl alcohol in an Erlenmeyer flask 100 c.c. of C. P. concentrated hydrochloric acid are added. The mixture is then quickly cooled to room temperature. The change in volume resulting from the mixture of hydrochloric acid and alcohol is disregarded. Immediately before using the reagent, 20 c.c. of diphenylamine (from Merck or G. Frederick Smith Co., melting point 52.5° to 53.3° C.) in absolute alcohol are added. The alcohol-diphenylamine solution is made by adding 20 c.c. of absolute alcohol to 3.4 Gm. of diphenylamine in an Erlenmeyer flask. The final reagent is conveniently made from this solution and the acid-alcohol mixture by pouring them back and forth a few times from one Erlenmeyer flask to the other.

When prepared as described here, the reagent gives an appreciably deeper color in the reaction with inulin than the reagent employed previously. In

\*From the Department of Medicine, University of Chicago.

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‡Some precautions and modifications have already been described.<sup>2, 4</sup>

order to keep the blanks as low as possible we prefer to prepare the required quantity of this reagent fresh each day.

*Removal of Fermentable Carbohydrate.*—Commercial inulin solutions, which are satisfactory for intravenous clinical use, contain appreciable amounts of a non-inulin fermentable material. Since this substance gives additional blue color with diphenylamine, it is essential that it be removed from both blood and urine samples by fermentation. This procedure should be employed even when Benedict's qualitative test for reducing substances is negative on the urine.

After fermentation of the samples of plasma or urine, and removal of yeast cells by centrifugation, only the clear supernatant fluid is used for preparing protein-free filtrates.

A special, starch-free yeast can be obtained from the Fleischmann Yeast Co. It is necessary to wash this yeast as originally described.<sup>2</sup> It is worth while to use the special, washed yeast because with it the blank values are markedly reduced. For example, we obtain blank values on plasma below 0.5 mg. per 100 c.c. inulin equivalent.

*Development of Color.*—Reaction tubes. Improved, pressure-resistant tubes, manufactured by Kimble Glass Co., have the following specifications: "Special, round bottom, screw cap vials, 19 to 20 mm. outside diameter and 110 mm. long; made of N-51A No. 2 weight glass; one end threaded to fit an 18 mm. G.C.A. cap, other end round bottom. Vials must have standard fire-finished flat tops. Glass must be evenly distributed; vials must be lehrred; lips must be square but not ground. Vials must have sandblasted spot as close to the shoulder as conveniently possible." New glass reaction tubes must be washed with soap and hot water before use in order to remove oily films from the glass. Then to remove traces of soap the tubes are rinsed thoroughly in hot water, filled with an acid-alcohol mixture similar to that described under "Reagents," stoppered, and heated in boiling water for at least fifteen minutes. After final rinsing with distilled water, these new tubes give satisfactory results. During subsequent use soap should not be employed; it is sufficient to clean the tubes by flushing with hot water, soaking in cleaning solution for at least one hour, and rinsing finally with distilled water.

*Rubber liners.* The use of replaceable, white rubber liners was recommended originally for the screw caps of the reaction tubes. The liners that we first obtained contained very little sulfur, which could easily be removed by boiling the liners in a dilute solution of sodium hydroxide. Liners must be free of sulfur which reacts with diphenylamine to produce a blue color similar to that given by inulin. When rubber liners are used, they must be cleaned in hot alcohol and rinsed in distilled water between determinations. If the glass reaction tubes or rubber liners are dirty, or are contaminated by traces of soap, the results will be less reproducible.

*Plastic liners.* It has become increasingly difficult to obtain durable rubber liners that are low in sulfur content. Because there is little prospect that this situation will improve, we have tried to use, with even more satisfactory results, 18-400 black phenol molded improved Lusterseal caps with glued-in Pliofilm pulp or waxed Vinylite pulp liners. The caps and liners do not have to be cleaned

before use. They must be discarded after being used once, but are cheap if purchased in quantity. Liners for this type of cap cannot yet be obtained separately. Satisfactory caps and liners are manufactured by Owens-Illinois Glass Co.\*

*Estimation of Color Intensity.*—A No. 635 filter is somewhat more suitable for measuring light transmission than the No. 660 filter employed originally.†

*Calibration.*—Standard inulin solutions may be kept for months without deterioration if the inulin is dissolved in saturated benzoic acid. A standard, equivalent in color development to a 1 per cent solution of inulin, may be obtained from the United States Standard Products Co., Woodworth, Wis. This concern has also developed a satisfactory preparation of inulin for clinical use.

*Determination of Blank Value of Reagents.*—When several sets of analyses are performed, three blanks on water plus reagents, omitting yeast, or three tubes containing inulin of known concentration plus reagents, should be heated in each water bath with the solutions of unknown concentration in order to determine the "center-point setting" of the galvanometer.

The "center-point setting" may be determined by means of an inulin solution of known concentration as follows: With the known solution in the Evelyn colorimeter, the galvanometer is adjusted to correspond to the previously determined reading of the known solution on the calibration curve. The known solution is then taken out, and the galvanometer reading without any tube in place is taken as the "center-point setting."

The "center-point setting" can be determined a little more accurately for unknown solutions of high inulin concentration (low galvanometer reading) by the simultaneous analysis of a known solution containing 3.5 to 4.5 micrograms of inulin per cubic centimeter; but for unknowns of low concentration the use of blank analyses on water plus reagents is preferable, and for making the original calibration curve, it is imperative.

## RESULTS

In analyzing inulin solutions of concentrations greater than 0.01 mg. per cubic centimeter, a small amount of blue precipitate is occasionally formed in the colorimeter tube. Ordinarily the error introduced by this precipitate is small, but the formation of the precipitate can be prevented by the proper dilution of samples before analysis. Whenever possible the unknown solution should be diluted to give a reading between 30 and 70 on the galvanometer scale of the Evelyn photoelectric colorimeter.

It must be emphasized that for most reproducible results the time of boiling should be standardized to exactly sixty minutes. The reaction is stopped by cooling the tubes immediately to room temperature. The colorimetric determinations should be carried out approximately thirty minutes after the tubes are removed from the boiling water bath.

\*Reaction tubes, caps, and liners may be obtained from regular laboratory supply houses. We obtain our supply through E. H. Sargent and Co., Chicago, Ill.

†Filter No. 635, supplied by Rubicon Co., Philadelphia, Pa., permits transmission of approximately 95 per cent of the light between 415 and 650 millimicrons, the approximate mean wave length of the transmitted band being 635 millimicrons.

In performing inulin clearances, the effect of analytical errors on the clearance values can be reduced to a minimum by heating corresponding plasma and urine samples together in the water bath, instead of analyzing plasma and urine samples at different times.

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## A CHEAPER NESSLER'S REAGENT BY THE USE OF MERCURIC OXIDE\*

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#### INTRODUCTION

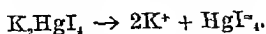
WITH the general rise in the price of chemicals at the present time, any economy in preparing large batches of Nessler's reagent should be of interest to most laboratories. Formation of the desired double halide from the oxide of mercury (and twice the usual equivalent of potassium iodide) is cheaper than its preparation from the very expensive mercuric iodide, or even from elementary iodine and mercury.

Nessler's reagent, as we all know, is essentially a strongly alkaline solution of a certain double iodide of potassium and mercury. Reaction with ammonia yields a colloidal<sup>1</sup> compound which in dilute solutions is evident as a yellow to brown color, and in less dilute ones (where the concentration of ammonia exceeds a few milligrams per hundred cubic centimeters) as a precipitate.

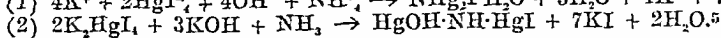
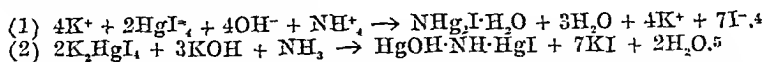
There is only slight doubt as to the composition of the effective double iodide in this old<sup>2</sup> and much-modified reagent. It is generally agreed to be formed from the equivalent of two moles of potassium iodide to each one of mercuric iodide:  $2KI \cdot HgI_2$  or  $K_2HgI_4$ . Friend<sup>3</sup> leaves the matter uncertain, but Treadwell and Hall<sup>4</sup> give the above salt in an equation for nesslerization. Mellor,<sup>5</sup> in his familiar *Comprehensive Treatise*, submits the usual mass of conflicting statements, but later (in his one-volume edition<sup>6</sup>) he also presents  $K_2HgI_4$  in an equation and states that the effective ratio of mercury to iodine should be 1:2.54, which corresponds exactly to the above compound. Yoe<sup>7</sup> likewise agrees, as do Latimer and Hildebrand.<sup>8</sup>

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Received for publication, July 14, 1941.

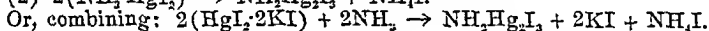
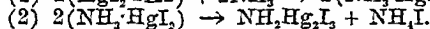
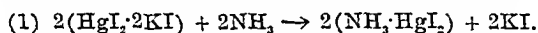
The double iodide evidently dissociates scarcely at all to give mercuric ions, for the addition of hydroxyl ions in considerable concentration may produce no precipitate of mercuric oxide. Ionization must largely occur thus:



In the presence of ammonium ion or ammonia as well as of alkali, this complex ion reacts to yield a peculiar, highly colored compound, probably a polymer. Equations such as these are given:



The chromatic substance, "mercuric hydroxiodamide"<sup>5</sup> or "oxydimercuric ammonium iodide,"<sup>16</sup> is of very doubtful composition. It is said<sup>4</sup> to be the iodide of "Millon's base," the white product obtained when aqua ammonia acts upon mercuric oxide. There is a host of formulas given for Millon's base and still more for Nessler's precipitate. Many texts are agreed that the latter compound has an *empirical* formula of  $NH_2Hg_2OI$ ,<sup>9</sup> which is variously written as:  $OH \cdot Hg \cdot NH \cdot HgI$ ,<sup>3</sup>  $NHg_2I \cdot H_2O$ ,<sup>4, 8, 22</sup>  $NH_2 \cdot Hg \cdot OHgI$ ,<sup>10</sup>  $HgOH \cdot NH \cdot HgI$ ,<sup>6</sup>  $HgO \cdot Hg (NH_2)I$ ,<sup>7</sup> etc. Franklin<sup>11</sup> in 1907 also gave  $Hg_2NH_2OI$ , but later<sup>12</sup> changed this to either  $2HgO \cdot NH_3 \cdot H_2O$  or  $4HgO \cdot 2NH_3 \cdot H_2O$ . The most recent attempts at analyzing this baffling compound were by Nichols and Willits,<sup>1</sup> who claim that it is  $NH_2 \cdot Hg_2I_3$ , and give this expression for nesslerization:—



The necessity for alkali is not obvious in their equations. The colored substance, the authors further state, is highly insoluble and forms an electro-negative suspensoid. This lyophobic nature is certainly indicated by the great gain in stability when soluble gums<sup>13</sup> are previously added to nesslerized solutions.

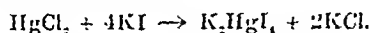
If we assume then that the effective double iodide is  $2KI \cdot HgI_2$ , and calculate the ratio of potassium iodide to mercuric iodide for various Nessler's reagents, we see that in the more satisfactory preparations this ratio, by design or by chance, closely approaches our theoretical ratio or slightly exceeds it for the sake of stability.  $2KI : HgI_2 = (2 \times 166.0) : 455.4 = \text{approximately } 332/455$ , or about 0.73.

| REAGENT               | REFERENCE | % KI  | % $HgI_2$ | RATIO | % NaOH     |
|-----------------------|-----------|-------|-----------|-------|------------|
| Frerichs and Mannheim | 14        | 2.5   | 3.5       | 0.71  | 15 (KOH)   |
| Folin and Denis       | 15        | 2.25  | 3.0       | 0.75  | 2.0        |
| Folin-Wu              | 16        | 1.125 | 1.50      | 0.75  | 7.0        |
| Bock-Benedict         | 17        | 7.0   | 10.0      | 0.70  | 10.0       |
| Koch-McMeekin         | 18        | 1.02  | 1.38      | 0.74  | 8.3        |
| Vanselow              | 19        | 3.49  | 4.55      | 0.77  | 11.2 (KOH) |

In fact, if one deviates very far from this figure, the sensitivity of the reagent decreases enormously. For example, when employing equal molarities of the potassium and mercuric iodides, a color will not develop with ammonia even when the reagent is strongly alkaline. It is desirable that there be just sufficient excess of potassium iodide to keep the mercuric iodide in solu-

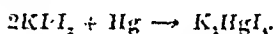
tion; but little more or sensitivity will suffer. Vanselow<sup>19</sup> recommends a 5 per cent excess of potassium iodide over that required to form the hypothetical  $K_2HgI_4$ , and thus the ratio in his reagent is 0.77. Other factors also reduce the sensitivity. With insufficient free hydroxyl ions, no color will develop at all, but great excess of alkali will produce a precipitate, probably mercuric oxide. Certain foreign salts, such as bicarbonates<sup>4</sup> or chlorides,<sup>15</sup> lower the intensity of the Nessler color. Temperature is also stated<sup>20</sup> to be rather influential.

The oldest method of preparing Nessler's reagent is the somewhat arbitrary one of adding an excess of potassium iodide to a saturated solution of mercuric chloride<sup>21</sup> (and then considerable base).

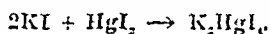


This technique is still given in many texts,<sup>9, 22</sup> but it is best suited for water analysis and such instances where the concentration of ammonia is quite low. Otherwise, the potassium chloride adds unwanted electrolyte which increases the tendency of the suspensoid to precipitate.

The time-honored procedure<sup>15, 16, 18</sup> of reacting metallic mercury with a tri-iodide solution yields the compound desired, but it is inconvenient and it appears that it is becoming obsolete.

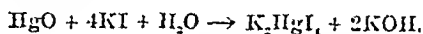


When mercuric iodide, free of such impurities as the sulfide and mercurous compounds, became readily available, the modification of dissolving mercuric iodide in strong, hot potassium iodide solution was adopted.<sup>16, 17</sup>



Dawson,<sup>23</sup> in his equilibrium researches, found evidence that in solutions where the dissolved mercuric iodide is relatively low, the chief complex electrolyte is  $K_2HgI_4$ .

The proposed method is this:



Here we have two end products, but both are needed for the reagent (the amount of alkali formed supplies only a fraction of that required). Rupp and Schirmer cited by Mellor<sup>5</sup> prepared the double iodide by the last reaction and proposed it as a method for standard alkali.

Friend,<sup>3</sup> and Latimer and Hildebrand,<sup>8</sup> also give the above equation as a reaction of mercuric oxide. This reaction was once used<sup>24, 25</sup> as a technique for providing standard alkali from a known weight of the pure oxide. Strangely, after a rather thorough search of the literature, I have encountered no mention of the utilization of this reaction of mercuric oxide to prepare Nessler's reagent.

#### PROCEDURE

Directions are given for the substitution of mercuric oxide in the preparation of the Koch-McMeekin<sup>18</sup> solution only, as this modification of the old Folin-Wu formula is probably the best Nessler's reagent as regards sensitivity, stability, and economy of materials that is in general clinical use. I have



prepared this formula (and others) by the authors' original method, with mercuric iodide, and with the oxide, and have found the three solutions apparently identical in excellence and composition.

Dissolve 19.1 Gm. of A.R. (analytical reagent quality) red mercuric oxide powder in a solution of 60 Gm. of A.R. potassium iodide in about 100 c.c. of ammonia-free water. Heat on an electric hot plate and stir until clear. The last bit of the solid dissolves with difficulty. Cool the deep yellow solution of the double iodide, make to 200 c.c., and then stir into 975 c.c. of pure 10 per cent sodium hydroxide. This 1,175 c.c. volume of the original Koeh-McMeekin reagent can be rather inconvenient. A liter could be prepared by using somewhat less of the iodide stock solution, thus: pour 160 c.c. of 50 per cent sodium hydroxide solution into about 600 c.c. of ammonia-free water. Add only 170 c.c. of the above iodide stock (with stirring) and dilute all to a liter with redistilled water. The objection may be raised that some of the iodide stock is left over. To avoid this, we can use a stock solution somewhat less concentrated than the original:

Dissolve 16.2 Gm. of A.R. red mercuric oxide in a solution of 51 Gm. of A.R. potassium iodide in 100 to 150 c.c. of ammonia-free water. Heat on an electric hot plate and add the powder in small portions, stirring until all is dissolved. Then dilute the double iodide solution thus obtained with about an equal volume of redistilled water and let cool. Dilute also 160 c.c. of clear (centrifuge if necessary) 50 per cent sodium hydroxide solution with about one-half liter of pure water. Pour in the iodide stock and make to one liter.

Red mercuric oxide is recommended since it costs somewhat less than the yellow form. The latter differs only in being of smaller particle size<sup>a</sup> and should be quite as satisfactory chemically, and perhaps more readily soluble.

How familiar is the phrase, "let stand to clear," but if the Nessler's reagent is prepared of pure materials, it is almost perfectly free of turbidity at once. Much purchased distilled water, especially that from raw river water, contains a surprising amount of ammonia and organic matter. If this is so, redistill that used for the reagent from an acid-permanganate solution. (In the interest of safety, add only enough potassium permanganate to maintain a decided purple color and just a small percentage of sulfuric acid.) Carbonate-free sodium hydroxide will be assured if one uses the convenient 50 per cent solution.

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# MEDICAL ILLUSTRATION

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## THE APPLICATION AND WEARING OF FACIAL PROSTHESES\*

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CARL DAME CLARKE, PH.D., BALTIMORE, MD.

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NUMEROUS articles have appeared in the medical and dental literature with regard to the manufacture of moulage prostheses or artificial parts to be worn on the human face and body. The most outstanding of these are by Kazanjian, Rowe, and Young,<sup>1</sup> Bereowitsch,<sup>2</sup> Zinsser,<sup>3</sup> Clarke,<sup>4, 5</sup> and Bulbulian.<sup>6, 7</sup> The majority of the prostheses considered in these articles were artificial noses, ears, cheeks, and eyes in which parts of the orbits and lids have been reconstructed rather than false teeth and gums that fit adjacent to mucous membrane. The prostheses to be discussed by me fit primarily next to skin surfaces and are held in place by mechanical devices, such as eyeglass frames, or an adhesive, such as gum mastic, dissolved in the proper solvent. The material already published did not explain in detail the actual wearing of prostheses. Instead, the manufacture of the prosthesis was considered, and methods were given for attachment by mechanical devices or by an adhesive. These articles left the prosthetist and his patient to determine by experimentation and experience the most comfortable adhesive mixture to use and the length of time required to rest the tissues. For example, some wearers of artificial parts on the face prefer to wear them continuously for a few days, others prefer to remove them every night. This depends largely on the extent of the restoration and the materials used.

I have been associated with two physicians who have had prostheses made months ago and are wearing them at the present time. Since these men themselves have facial restorations, they are exceptionally well qualified to know from firsthand experience the problems that arise during the application and wearing of prostheses. Furthermore, as physicians they understand the nature of any irritations that may begin and know how to correct them. One of these cases represents a partially restored nose that was required after the removal of an epithelioma; the other is a partially restored ear designed to correct a defect resulting from an accident. I am, therefore, indebted to these two men, who prefer to remain anonymous, for the following information and description of their actual experiences during the wearing of their respective prostheses.

### PRECAUTIONS TO BE TAKEN IN MANUFACTURE OF PROSTHESES

In the manufacture of a prosthesis it is essential to use the utmost care in the preparation of both surfaces of the material, that is, the outer or visible surface and the surface that will come in contact with the skin. The exposed

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\*From the Department of Art, University of Maryland School of Medicine.

contours should be regular, smooth, and lifelike, with the correct proportions and color. The contact surface should be smooth, without sharp points or granular areas that do not make complete contact. It must fit snugly and completely to the skin on which it will be attached; any irregularity or depression in the skin must be filled with a corresponding negative impression on the moulage. It is for this reason that flexible materials, such as latex and gelatin-glycerin-sorbital mixtures are more practical than the hard, firm, unyielding substances. Metal, vulcanite, and the synthetic resins, such as vernonite and vinyl acetate, belong to the latter group.

Surface area should not be sacrificed for cosmetic effect in preparing the contacting surface. In other words, the surface area should be sufficiently extensive to approximate a considerable portion of the skin area when applied to it with the adhesive substance; otherwise the border of the prosthesis will not approximate closely the dermal structure. The edges will be more apparent and fixation will be unsatisfactory. This statement from the wearer of a prosthesis came as a surprise, because I labored under the assumption that the smaller the skin surface covered by an artificial part, the more realistic the effect. In his opinion sufficient skin surface must be covered to make the artificial part feel comfortable and at the same time give complete assurance that it will not fall off. Naturally, this matter should be tempered according to the size and weight of the prosthesis and the strength of the adhesive used. When making a prosthesis, the seam or junction between the natural and artificial skin surfaces should come at points, lines, or areas that will prevent detection as far as possible. Folds and character lines within the skin surface offer the best areas for forming the joining lines. When such details are considered, the moulage maker may take advantage of them in making the prosthesis to produce surfaces that will support the weight of the prosthesis and at the same time escape detection.

#### ADHESIVE MATERIALS

Bereowitsch<sup>2</sup> and Zinsser<sup>3</sup> advised the use of gum mastie dissolved in alcohol as an adhesive to hold a nose or an ear prosthesis to the skin surface. In trying this mixture I found that the alcohol dried too slowly from the mastie to effect satisfactory adhesion. Furthermore, the mastie turned milky in the presence of moisture, an effect known as *bloom* in the field of art. Since gum dammar does not react in this manner, I substituted it for the mastie.

Gum dammar and gum mastie possess the following qualities: (1) They are transparent when in solution. (2) They are insoluble in water. (3) They are non-irritating. However, I have heard of one case in which the gum mastie solution was blamed for causing a skin rash at the place where the prosthesis joined the skin. I am inclined to believe that this was the result of an idiosyncrasy to the gum, or that there was a mechanical irritation created by removing the part too frequently. In numerous other instances the gum mastie was used properly over a period of years without any detrimental effect. (4) An excess of either gum dammar or gum mastie or any quantity of these which remains dried on the skin or the prosthesis may be removed with a 70 per cent solution of alcohol. (5) These ingredients are inexpensive, a year's supply costing only a few cents.

(6) Both gum mastie and gum dammar are not affected by normal temperature changes. Gum dammar is not affected by excess humidity, and gum mastie is not likely to be changed to any great extent. The wearer may notice that the gum mastie will bloom when it is applied in rainy weather. However, this is of little consequence. (7) A normal prosthesis may be attached by the use of these materials in a few minutes, fifteen minutes at the most.

To overcome the slow drying of the alcohol, as recommended by Bereowitsch and Zinsser, I used a mixture of equal parts of alcohol and ether in which the gum dammar was dissolved. The patient was advised to paint the gum dammar solution on the surface of the prosthesis that was to come into contact with the skin surface, then put the prosthesis in place and hold it for a few minutes until complete and comfortable adhesion was effected. This required only a few minutes. I instructed the patient to increase the amount of ether in the solution if he wanted quicker adhesion, and to add more alcohol if the adhesion took place too quickly or dried before the prosthesis could be put in place.

This reasoning seemed logical to me, but I found that one wearer preferred to use ether entirely as a solvent for gum mastie. The fact that this gave satisfactory results over a period of years speaks for itself. The wearer claims that the gum mastie is more effective when dissolved in ether, and if kept in solution will prove a more uniform and adequate adhesive agent than if prepared for each individual application. It is best applied to the prosthesis by means of a medicine dropper, and the density of the solution should be such that it can be drawn into the dropper with ease. The ether evaporates very readily from the solution if the container is left open for any length of time and should be replaced from time to time if the mixture becomes too thick. In the event the solution becomes too thin, additional gum should be added. I always prepare a bottle of the gum solution and give it to the patient with his prosthesis. Instead of the medicine dropper, I use a bottle with a stopper that contains a small bristle brush. This brush is used to apply the adhesive to the contacting surfaces. After the adhesive is applied, the prosthesis is put in place and held with firm, constant, and regular pressure until the solvent evaporates from the gum and complete adhesion takes place.

#### WEARING OF THE PROSTHESIS

The prosthesis, after its application, can usually be worn for about three days. The gum mastie then becomes only loosely adherent and removal at that stage is simple, only a small amount of traction being necessary. If the prosthesis is removed at such time there will be no skin abrasions or irritations. The prosthesis may then be cleaned with alcohol and replaced. There is no reason why a prosthesis made of latex or a gelatin-glycerin composition should not be worn at night. As a matter of fact, it is unwise to detach it too frequently, particularly when it is attached firmly to the skin. Of course, the size of the prosthesis and the material from which it is made may have some bearing on these instructions. If the prosthesis is attached to the eyeglass frame it may be possible to dispense with an adhesive altogether. This depends upon the location and extent of the lesion and the size of the prosthesis.

## CARE OF THE SKIN

When the prosthesis is removed, the dried nonadhesive debris of the gum-solvent solution should be cleaned away carefully with a 70 per cent alcohol solution. Gentle massage with a soft cloth dampened with alcohol is all that is necessary. The generous application of alcohol to the surrounding skin surface with the fingers will be found comforting and helpful. It will also be found practical and wise to leave off the prosthesis occasionally for several hours, as during sleep. The application of tincture of green soap before this replacement is made may aid in protecting the surface.

In a recent communication one of these physicians supplied the following information: "I have been using the monlage prosthesis for two years and during this period have had absolutely no skin or other reaction of any sort in spite of the fact that my skin is unusually sensitive. My prosthesis is most satisfactory and is not noticed by the public; however, upon a few occasions some of my medical friends have asked me who did the plastic operation and were wondering from what area the skin was removed. It has been my choice to remove it each night, replacing it in the morning, which is done in less than a minute's time."

If the principles here outlined are followed carefully, a satisfactory wearing of the prosthesis will be experienced with the least amount of physical and mental discomfort and the maximum in cosmetic efficiency.

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 Erratum

On page 1799 of the August number of the JOURNAL in the article by Theodore S. Rosen, M.D., and George W. Lubinsky, A.B., entitled "Freezing of Tissues With 'Dry Ice' for Microtome Sectioning of the Entire Brain," the first two sentences in the paragraph under "Freezing Apparatus" should read: "The freezing attachment consists essentially of (1) a metal plate upon which the slab of tissue is frozen, and (2) a means of keeping the 'ice' in firm contact with the metal plate. The plate is cooled by the 'ice' and in turn chills the tissue, at the same time anchoring the tissue to the plate." The words that are italicized were omitted from the text.

# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**PNEUMOCOCCI, Sensitivity of to Sulfapyridine, A Rapid Qualitative Test for Resistance,** Moore, F. J., Thomas, R. E., and Hoyt, A. J. A. M. A. 117: 437, 1941.

Each of 4 mice was inoculated intraperitoneally with 0.2 c.c. of 10 per cent sulfapyridine suspended in 20 per cent acaia. Sputum (or pus) was made grossly homogeneous and liquid by mixing with a minimum necessary amount of sterile saline solution. Within the first two hours after treatment, 4 treated and, in addition, 3 untreated mice were alternately inoculated with the sputum mixture, as large a volume as possible (up to 1 c.c.) being used for each mouse. At various intervals thereafter peritoneal exudate was removed from each mouse and examined rapidly to determine the number and the morphology of the pneumococci. Efforts to make accurate counts were avoided. The test was read when growth of pneumococci in the untreated mice had become unquestionable (as indicated by hundreds per oil-immersion field), and at no earlier time. If, at this time, the treated mice showed no increase in the number of pneumococci, or if there had been scanty growth with a preponderance of atypical forms, the strain was called sensitive; if smears of treated and control mice were virtually indistinguishable, the strain was called resistant (fast or refractory).

**TRICHINOSIS,** Wyrens, R. G., Tillisch, J. H., and Magath, T. B. J. A. M. A. 117: 428, 1941.

In an analysis of 19 cases of clinical trichinosis and 21 cases of asymptomatic infection incidental diagnoses were made during routine examination of surgical pathologic material. Included in the series are 2 cases suggestive of repeated infection.

Asymptomatic infection with *T. spiralis* and clinical trichinosis are two conditions which should be clearly separated.

**SPOTTED FEVER, Rocky Mountain,** Hutton, J. G. J. A. M. A. 117: 413, 1941.

The literature shows that cases of Rocky Mountain spotted fever have been reported from thirty-seven of the forty-eight states.

Rocky Mountain spotted fever is transmitted to human beings through infected ticks, and the causative organism is *Dermacentor rickettsii*.

Three types of the disease are recognized: the mild, the severe, and the fulminating. The cutaneous manifestations of Rocky Mountain spotted fever are characteristic.

A drug exerting any great amount of specific action against this infection has not been found.

Vaccine gives partial protection for a period of one year or less.

The United States Public Health Service vaccine for Rocky Mountain spotted fever is prepared at the Rocky Mountain Laboratory of the National Institute of Health at Hamilton, Mont.

The vaccine is given in two doses of 2 c.c. each at an interval of five days, either subcutaneously or intramuscularly. The known data indicate that the vaccine fully protects the majority of persons against the less virulent strains but that the average person is only partially protected against the highly fatal type of Rocky Mountain spotted fever, and that the maximum degree of protection conferred is retained for less than one year.

**GNOCOCCIC INFECTIONS, Cutaneous Diagnosis of, Corbus, B. C., and Corbus, B. C., Jr.**  
J. A. M. A. 116: 113, 1941.

A person infected with a specific organism is in a state of hypersensitivity to that organism as long as it remains within the body.

In a control series of 100 persons known not to be infected with gonococci, 85 per cent gave negative reactions to the cutaneous test described.

According to the authors' experience, this test as a diagnostic adjunct in gonococcal infections is more valuable than any other procedure previously available.

The antigen consists of 0.1 c.c. of a standard bouillon culture filtrate with 0.1 c.c. of bouillon as a control.

**COLOR INDEX, A Simple Hemoglobin-Red Cell Ratio to Replace the, Isaacs, R. J. A.**  
M. A. 116: 2258, 1941.

A simple relationship between the hemoglobin content of the blood and the number of red blood cells may be expressed by this formula for "normal": Three times the number of grams of hemoglobin per hundred cubic centimeters divided by the number of hundreds of thousands of red blood cells per cubic millimeter equals 1. It is distinctly less than 1 in hypochromic anemias and more than 1 in hyperchromic conditions.

**URINE, Method for Rapid Alkalinization of, Bick, M., and Drevermann, E. B. M. J.**  
Australia 1: 223, 1941.

The authors advocate the immediate intravenous administration of 20 c.c. of the sodium lactate-sodium bicarbonate solution on the first appearance of any symptoms suggestive of the occurrence of hemolysis after blood transfusion. This is, however, only the immediate treatment, and as large quantities of fluid are required to maintain the diuresis, the purpose of the injection is to alkalinize the urine rapidly in order to permit time for the setting up of a continuous intravenous infusion of isotonic sodium lactate solution.

**HEMOPHILIA, Diagnosis of, Quick, A. J. Am. J. M. Sc. 201: 469, 1941.**

The blood of a true case of hemophilia should show the following:

1. Coagulation time (Lee White test), over eight minutes at 37.5° C.
2. Coagulation time of recalcified plasma:
  - a. High speed centrifugation, over five minutes.
  - b. High speed centrifugation, over three minutes.
  - c. Clotting time should decrease on standing.
3. Prothrombin concentration (Quick's method), above 70 per cent.
4. Bleeding time (Duke's method), not over four minutes.
5. Clot retraction, not over sixty minutes after coagulation occurs.
6. Tourniquet test (Rumpel-Leede technique), not over four petechia in specified area.

**BLOOD DONORS, Response of, to Iron, Santy, A. C. Am. J. M. Sc. 200: 790, 1941.**

The hemoglobin response of 27 active professional blood donors was studied under iron therapy and without medication.

It was found that hemoglobin regeneration after donations took place eight times more rapidly when iron was given.

The return to the previous level was complete in an average of eleven days after a 500 c.c. donation under iron therapy, whereas a far longer period was necessary when iron was withheld.

A rough calculation of the amount of hemoglobin in the total circulation revealed the fact that the average active donor in this series could not maintain a positive balance without iron medication.



It was found that the response to iron usually continued after its withdrawal, indicating that the most effective time to administer iron is before blood loss.

It is concluded that the diet of regular donors should be supplemented with iron. Ferrrous sulfate, because of its convenience and freedom from reactions, was regarded as a very satisfactory form of iron for blood donors.

**"STORED" BLOOD.** Effect of Glucose in the Preservation of Citrated Human Blood Stored at 4-6° C., Belk, W. P., and Rosenstein, F. *Am. J. M. Sc.* 200: 841, 1941.

Glucose (5.4 per cent) prevents the hemolysis of erythrocytes of whole citrated human blood kept at 4° to 6° C. for three to four weeks. With citrate alone hemolysis appears near the end of the first week.

This preserving effect is less marked on granulocytes, platelets, and on the clot-forming properties.

One part of isotonic glucose to two parts of blood is effective, and has the advantage of increasing not too greatly the amount of fluid to be injected.

It must not be thought that blood preserved by chilling and the addition of glucose remains in a state of hibernation, or suspended animation. The processes of decay go on steadily, and are retarded only by the favorable environment. There is, therefore, a strict limit to the time that blood for transfusion may properly be stored. This limit is shorter than is generally supposed due to the fact that these red blood cells become incapable of substituting for the cells of the recipient prior to suffering any hemolysis.

**"STORED" BLOOD:** Survival Time of Erythrocytes of Citrated Human Blood Stored at 4-6° C., Belk, W. P., and Barnes, B. C. *Am. J. M. Sc.* 200: 838, 1941.

The erythrocytes of citrated human blood kept at 4° to 6° C. were found to survive in recipients as long as the cells of fresh blood when storage time did not exceed two or three days. After this they disappeared with increasing rapidity as storage time increased, until, after seven or more days in the "bank" their post transfusion survival was in no case longer than twenty-four or forty-eight hours.

**GONOCOCCUS, Preservation of, in Frozen Urines and Broth,** Wortman, M. S., Gronau, A., Deakin, R., and Love, F. *Ven. Dis. Inform.* 22: 195, 1941.

#### *Routine Method:*

The urethral discharge of male patients with gonorrhea was collected on a sterile swab which was then immersed in a serologic tube containing 1.5 c.c. of infusion broth. Subsequently the specimen was plated out on a chocolate agar plate. If there was no discharge, the first portion of the urine was collected in a sterile centrifuge tube. The sediment was taken up on a swab after centrifugation and transferred into a broth tube. A culture was then made as stated above.

In female patients the cervix was exposed by means of a speculum, avoiding the use of lubricants. A specimen was taken from the urethra and cervix with a swab which was then immersed in a broth tube and later plated out as described.

#### *Freezing Method:*

(A) *Broths.* A routine culture was made from the discharge in both male and female patients, and the swab was then placed back into the broth tube. The broth tube was immersed in the freezing mixture of dry ice and alcohol (temperature about -72° C.). Freezing occurred in about fifteen to twenty seconds. The specimens were transferred when frozen to a thermos bottle containing dry ice.

(B) *Urines.* Urines collected in sterile 15 c.c. centrifuge tubes were frozen by immersing the tubes in the freezing mixture. Freezing occurred in about one and one-half to two minutes. The tubes were transferred after freezing to a thermos bottle containing dry ice.

**TUBERCLE BACILLI, Demonstration of, Sasano, K. T., Caldwell, D. E., Needham, E. L., and Medlar, E. M.** *Am. Rev. Tuberc.* 43: 263, 1941.

A comparison of results obtained from culturing and inoculating guinea pigs for the detection of tubercle bacilli in 1,270 specimens from human sources is presented. The results obtained from culturing the spleens from 100 consecutive tuberculous guinea pigs, using the technique briefly outlined in the text, are given. The advantages and disadvantages of each method are discussed. To substitute the cultural method for the guinea pig inoculation procedure seems inadvisable. The utilization of both methods counterbalances certain inherent disadvantages in each. As a routine procedure a combination of the two methods will make possible a more accurate detection of tubercle bacilli present in small numbers than is possible where only one or the other method is used.

**TUBERCLE BACILLI, Culture of, Steenken, W., Jr., and Smith, M. M.** *Am. Rev. Tuberc.* 43: 309, 1941.

A fifty-hour sputum specimen is collected in a 100 c.c. wide-mouthed bottle, stoppered with a good grade of cork. An equal volume of 4 per cent sodium hydroxide is added, and the mixture is homogenized for ten minutes in a shaking machine, followed by one hour's incubation at 37.5° C. It is then centrifuged for ten minutes at high speed in sterile pyrex tubes which have been sealed with sterile rubber caps (type used for capping babies' milk bottles). The sediment is neutralized with normal hydrochloric acid containing 10 per cent by volume of phenol red indicator, and adjusted on the acid side to a pH of about 6.4 (it is best to approach the final pH with either N/10 hydrochloric acid or N/10 sodium hydroxide as may be required).

This suspended sediment is divided into three equal portions. Two of these portions are transferred to two small vials with round bottoms that will stand centrifuging and which can be stoppered with small corks. (Small round bottom 5 c.c. vaccine vials are well suited for this purpose.) To one vial, 5 c.c. of a 1:1,000 solution of malachite green is added, and to the other vial, 5 c.c. of a 1:10,000 solution. The third vial is kept as a control. The vials which contain the dye are then stoppered and agitated in the shaking machine for ten minutes, then centrifuged for ten minutes at high speed. The supernatant fluid is pipetted off with a sterile capillary pipette. The sediment is then suspended in 1 c.c. of sterile distilled water.

**STREPTOCOCCI, Comparison of Beta-Hemolytic on Maltose-Blood-Agar. A Presumptive Test for Group A, Simmons, R. T., and Wilson, H. M. J.** *Australia* 1: 74, 1941.

A total of 869 strains of beta hemolytic streptococci of human origin of groups A, B, C, and G were examined on maltose-blood-agar plates as a presumptive means of distinguishing strains of group A from the other three groups, which are commonly found in man but are rarely pathogenic to man.

Of 500 strains of group A streptococci, all but four (0.8 per cent) were recognized. Of 369 strains of groups B, C, and G, all but 15 (4 per cent) were recognized as not of group A.

As a presumptive test for group A streptococci, the medium maltose-blood-agar was useful, and, with few exceptions, reliable. As a presumptive test for group B among hemolytic streptococci of human origin, it was quite reliable. Groups C and G were not distinguishable from one another, but most strains of groups C and G were distinguishable from group A, and all strains were distinguishable from group B.

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## *CLINICAL AND EXPERIMENTAL*

### AMPHETAMINE SULFATE-ETHYL ALCOHOL ANTAGONISM IN THE RABBIT\*

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**D**URING an investigation in man of the stimulating action of amphetamine (benzedrine) sulfate on abnormal mental states characterized by depression, certain toxic depressive states caused by alcohol were included, and it was observed that the alcoholic cases were among those most favorably affected.<sup>1-4</sup> These observations indicated that in man there is an antagonism between amphetamine sulfate and ethyl alcohol.

In a series of over 100 cases of alcoholism, with and without psychosis, our most prominent results of treatment included: (1) a reduction by one-half in the length of time for recovery of the acute alcoholic psychoses; (2) a slight increase in the number of recoveries; (3) a diminution in the severity of the psychotic manifestations; (4) a dissipation of the "hang-over" effects of inebriation; (5) an unsatisfactory response in chronic alcoholic addiction; (6) a differentiation between uncomplicated alcoholic depressions and psychogenic depressions masked with alcohol by the rapid response of the former; and (7) an antagonistic or "sobering" effect of amphetamine during acute alcoholic intoxication. The latter was most striking. Boisterous, excited, hyperactive, surly and irritable individuals became quieted; some fell asleep after the medication. Tremor in these patients was aborted. Occasionally, however, the excitement and the tremulousness was increased. The incoherence and incoordination of more profound inebriation was rapidly replaced by a more coordinate state. Persons who had imbibed sufficiently to become stuporous frequently were aroused and rational within thirty minutes following the intravenous injection

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of 20 to 30 mg. of amphetamine sulfate. The drug rendered depressed, sullen, and asocial intoxicated individuals more cheerful and adaptable. Some chronic alcoholic patients took amphetamine before drinking in order to consume larger quantities of alcohol without the appearance of unpleasant symptoms. We concluded from these investigations that in man there is an antagonism between amphetamine sulfate and alcohol, which is of greatest value clinically in the more acute phases of alcoholism with and without psychosis.

#### PRESENT INVESTIGATION

Because of the satisfactory response of acutely alcoholic patients to amphetamine sulfate, it seemed desirable to test in animals the limits of the antagonism between the two drugs. Since this investigation was begun (October, 1938), Werner<sup>5</sup> has published a brief report on the effects of amphetamine sulfate on alcoholic depression in animals. He stated that 7 c.c. per kilogram of alcohol orally protected rabbits against the lethal effects of subsequently administered amphetamine sulfate. The lethal dose of amphetamine was increased approximately 1.5 times. The drug did not completely awaken the depressed animals. However, in rabbits receiving 2.5 c.c. per kilogram of alcohol intravenously, coordination of the hind legs was restored by amphetamine. Entirely similar results were obtained by Werner with pyridine- $\beta$ -carboxylic acid diethylamide (coramine), metrazol, and picrotoxin.

#### MATERIALS AND METHODS

Inasmuch as both the alcohol and the amphetamine sulfate had been administered orally in the clinical studies, it was decided to administer the drugs to animals by the same route. The rabbit was used because of suitable size for ease in passing a stomach tube. From a colony of about 800 rabbits, approximately 600 were chosen at random and employed in the present investigation. These animals were equally distributed in sex, and varied in age from 6 to 15 months, and in weight from 1.50 to 3.20 kg. Subsequently, the experimental group was reduced to 423 by eliminating from the data those rabbits weighing less than 1.70 or more than 3.00 kg., because of the observation of Ehrlich, Levy, and Krumbhaar<sup>6</sup> that the minimum lethal dose varied with the weight (age) of the animals and that the heavier animals were more susceptible. However, in no instance were the results modified by thus restricting the number of animals. No seasonal variation in the susceptibility of the animals to the same mixture of the drugs was observed. In many instances the solutions containing different mixtures were employed simultaneously, thus rendering the variations in response more easily comparable.

All animals were fed on a standard laboratory diet, which had maintained in good health for several years the remaining 200 rabbits (employed in pregnancy tests). In every experiment food was withheld routinely for more than sixteen hours before and after medication. No animal was used more than once throughout the investigation. All medications were administered intragastrically as solution, using an 18 F urethral catheter as an esophageal tube. Preliminary experiments indicated that the average stomach capacity of the selected animals was 150 to 250 c.c. All solutions were devised, therefore, so

that the total volume of a single dose never exceeded 150 to 175 c.c. (depending on the size of the animal). All solutions were made, however, so that the volume administered would be as large as possible, in order to minimize drug loss and in order to eliminate the necessity of washing down a given dose with additional fluid and thus changing the concentration. To secure uniform freshness, the solutions were prepared as needed. The amphetamine sulfate used was the racemic compound, in the form of a powder,\* dissolved in distilled water. The alcohol solutions were prepared from alcohol U.S.P. (i.e., 95 per cent).

The particular techniques are described in connection with the divisions of the problem. In the lethal dose experiments the dose was considered to be fatal only if the animal died within fourteen days of medication. The term "minimum lethal dose 50 (M.L.D. 50)" is used to describe that dose which was lethal to at least 50 per cent of the animals to whom it was given.

TABLE I

ANALEPTIC EFFECT OF AMPHETAMINE SULFATE ON NARCOSIS OF MODERATE AMOUNTS OF ETHYL ALCOHOL

| CONTROL GROUP<br>5 GM. ALCOHOL PER KG. |                                    |                                       | TEST GROUP<br>5 GM. ALCOHOL PER KG. AND<br>85 MG. AMPHETAMINE PER KG. |                                    |                                       |
|--|------------------------------------|---------------------------------------|---|------------------------------------|---------------------------------------|
| ANIMAL<br>NUMBER                       | ONSET OF<br>NARCOSIS<br>IN MINUTES | DURATION OF<br>NARCOSIS<br>IN MINUTES | ANIMAL<br>NUMBER  | ONSET OF<br>NARCOSIS<br>IN MINUTES | DURATION OF<br>NARCOSIS<br>IN MINUTES |
| F3                                     | 27                                 | 293                                   | F25   | 77                                 | 275                                   |
| F6                                     | 10                                 | 340                                   | F26   | 57                                 | 353                                   |
| F9                                     | 20                                 | 290                                   | F28   | 54                                 | 320                                   |
| F12                                    | 17                                 | 520                                   | F29   | 45                                 | 280                                   |
| F13                                    | 15                                 | 425                                   | F30   | 81                                 | 257                                   |
| F15                                    | 23                                 | 485                                   | F31   | 69                                 | 251                                   |
| F17                                    | 26                                 | 354                                   | F32   | 32                                 | 345                                   |
| F19                                    | 14                                 | 463                                   | F34   | 41                                 | 345                                   |
| F20                                    | 15                                 | 344                                   | F35   | 30                                 | 294                                   |
| F21                                    | 12                                 | 345                                   | F36   | 57                                 | 264                                   |
| F22                                    | 8                                  | 479                                   | F37   | 53                                 | 295                                   |
| F23                                    | 18                                 | 461                                   | F38   | 57                                 | 363                                   |
| F24                                    | 22                                 | 505                                   | F40   | 74                                 | 349                                   |
| Total                                  | 227                                | 5304                                  |   | 727                                | 3991                                  |
| Average                                | 17.4                               | 408                                   |   | 55.9                               | 307                                   |

## RESULTS

*A. Moderate Dose Experiments.*—The following experiments were performed to determine the effect of amphetamine sulfate on the narcosis of moderate amounts of ethyl alcohol (Table I):

(1) To a series of 13 animals (control group), 5 Gm. per kilogram of alcohol were administered intragastrically. The onset and the duration of narcosis were recorded. The animals were stimulated at five- to ten-minute intervals by a forceful pinching of the ear with a mouse-toothed forceps, and were not considered to be in a state of narcosis if they could coordinate their hind legs sufficiently to hop once without losing balance. Narcosis occurred in 17.4 minutes (average) after the administration of the alcohol, and lasted 408 minutes (average).

\*Racemic amphetamine sulfate powder was supplied through the courtesy of Mr. Theodore B. Wallace, Smith, Kline and French Laboratories, Philadelphia, Pa.

(2) The same experiment was performed in another series of 13 animals (test group) to whom 5 Gm. per kilogram of alcohol and 85 mg. per kilogram of amphetamine sulfate were given in the usual manner. Sleep ensued in 55.9 minutes (average) and lasted 307 minutes (average).

Comparison reveals that the addition of the amphetamine sulfate delayed the onset of the narcosis about 200 per cent, and shortened it about 25 per cent.

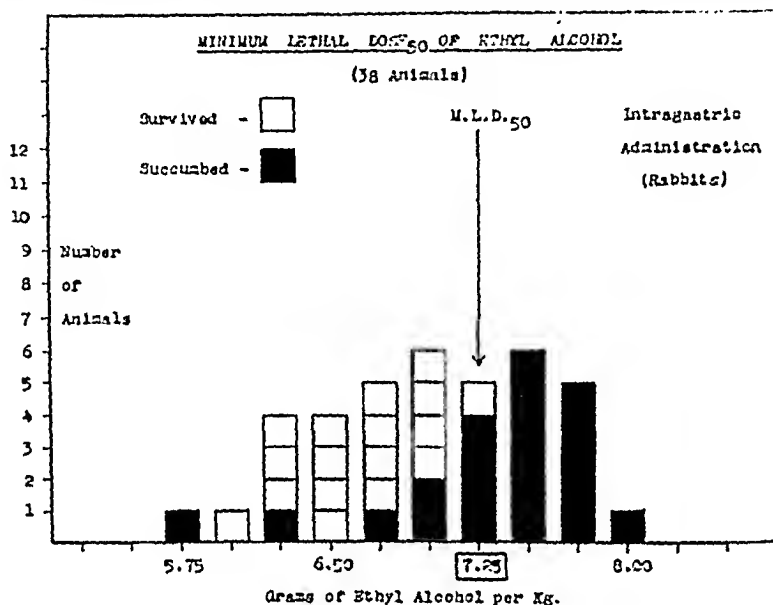


Fig. 1.

*Statistical Treatment of Results: Onset of narcosis*—the standard error of the mean of the control group was 1.786 minutes and of the test group, 4.94 minutes. The ratio of the difference of the means to the standard error of their difference was 1.857, indicating but thirty-two chances in one thousand of a reversal in similar experiments. *Duration of narcosis*—the standard error of the mean of the control group was 7.869 minutes and of the test group, 12.9 minutes. The ratio of the difference of the means to the standard error of their difference was 11.5, indicating an infinitesimal chance that there would be a reversal in similar experiments. The degree of significance of the changes of the onset and the duration of the narcosis is increased by the fact that amphetamine sulfate altered both of these factors in the same direction.

*B. Lethal Dose Experiments.*—Five experiments were performed to determine the effect of amphetamine sulfate on lethal doses of ethyl alcohol, and the effect of ethyl alcohol on lethal doses of amphetamine sulfate:

(1) *M.L.D. 50 of Ethyl Alcohol:* Varying doses of 25 per cent ethyl alcohol solution were given to 38 animals, and the number of deaths within fourteen days was recorded. The volume of the solution administered varied from 58 to 112 c.c. The M.L.D. 50 was found to be 7.25 Gm. of alcohol per kilogram (Fig. 1).

(2) *M.L.D. 50 of Amphetamine Sulfate:* In a manner similar to the preceding experiment, 0.25 per cent solution of amphetamine sulfate was given to 68 animals. The volume of the solution administered varied from 44 to 130 c.c. The M.L.D. 50 was found to be 85 mg. per kilogram (Fig. 2).

(3) *M.L.D.*<sub>50</sub> of Mixture A: Mixture A (7.25 Gm. of ethyl alcohol (1 *M.L.D.*<sub>50</sub>) and 42.5 mg. of amphetamine sulfate ( $\frac{1}{2}$  *M.L.D.*<sub>50</sub>) in 37 c.c.) contained alcohol and amphetamine in approximately 25 per cent and 0.15 per

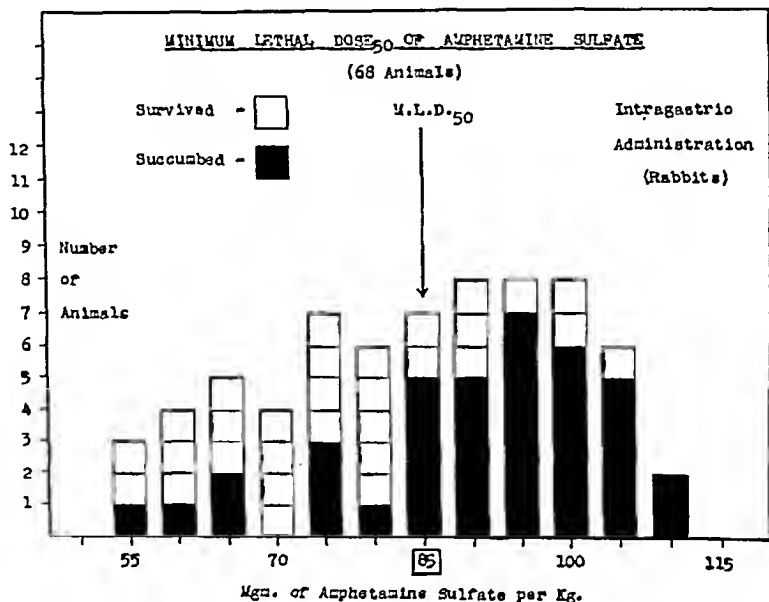


Fig. 2.

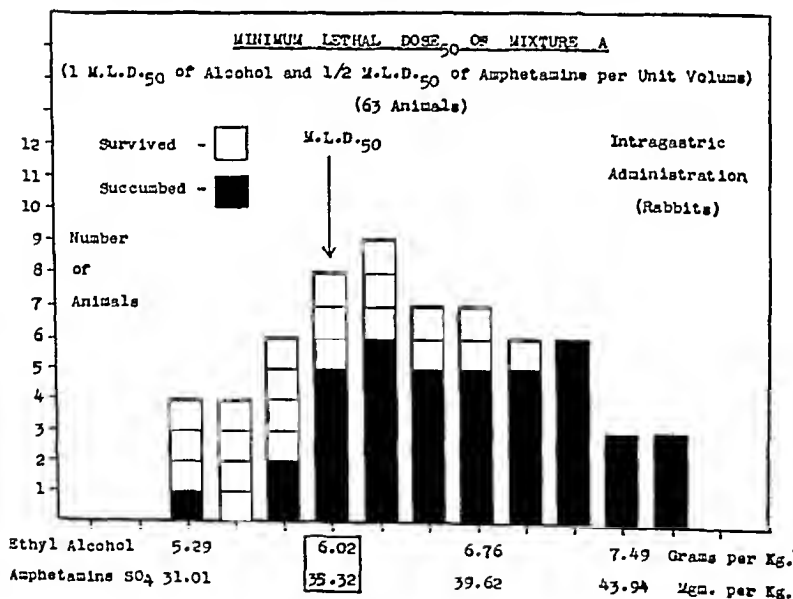


Fig. 3.

cent concentration, respectively. Varying quantities of this mixture were administered to rabbits, and the number of deaths within fourteen days was tabulated. The amounts of ethyl alcohol per kilogram and amphetamine sulfate per kilogram for a given quantity of mixture A solution were then calculated.

The volume administered varied from 45 to 115 c.c. A series of 63 animals was required. The M.L.D.<sub>50</sub> of the mixture A solution was found to contain 6.025 Gm. per kilogram of ethyl alcohol and 35.32 mg. per kilogram of amphetamine sulfate (Fig. 3).

(4) *M.L.D.<sub>50</sub> of Mixture B*: The procedure was the same as the preceding one. Mixture B (7.25 Gm. of ethyl alcohol (1 M.L.D.<sub>50</sub>) and 85 mg. of amphetamine sulfate (1 M.L.D.<sub>50</sub>) in 37 c.c.) contained alcohol in approximately 25 per cent solution, and amphetamine in approximately 0.3 per cent solution. A volume of solution varying from 47 to 102 c.c. was given to 55 animals. The M.L.D.<sub>50</sub> of the mixture B contained 5.78 Gm. per kilogram of ethyl alcohol and 67.72 mg. per kilogram of amphetamine sulfate (Fig. 4).

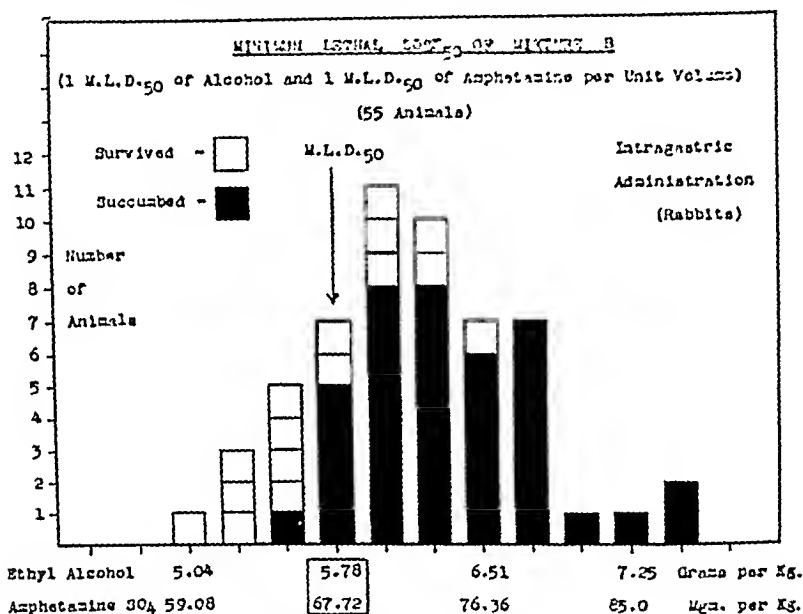


Fig. 4.

(5) *M.L.D.<sub>50</sub> of Mixture C*: The same technique was employed. Mixture C (3.625 Gm. of ethyl alcohol ( $\frac{1}{2}$  M.L.D.<sub>50</sub>) and 85 mg. of amphetamine sulfate (1 M.L.D.<sub>50</sub>) in 34 c.c.) contained alcohol in approximately 16 per cent solution, and amphetamine in approximately 0.25 per cent solution. A volume of solution varying from 62 to 175 c.c. was administered to 99 animals. The M.L.D.<sub>50</sub> of the mixture C solution was found to contain 5.239 to 5.968 Gm. per kilogram of ethyl alcohol and 125 to 140 mg. per kilogram of amphetamine sulfate (Fig. 5). It should be noted that even in this large series of animals, there was so much individual variation that an exact single value for the M.L.D.<sub>50</sub> could not be determined. Inasmuch as the range of values obtained was satisfactory to indicate the trend, the experiments were terminated.

#### ANALYSIS OF RESULTS AND DISCUSSION

The results of animal experimentation cannot be strictly applied to man. However, in the experiments herein recorded, amphetamine sulfate has exhibited



in antagonistic effect in rabbits intoxicated with moderate quantities of alcohol, and this seems to confirm the clinical observations previously cited of the effectiveness of amphetamine sulfate in acute alcoholic states in man.

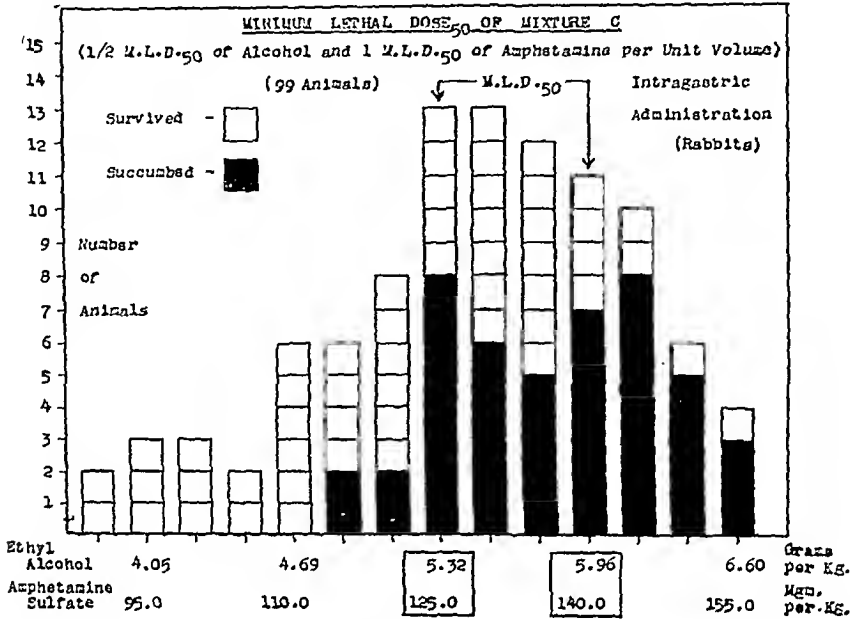


Fig. 5.

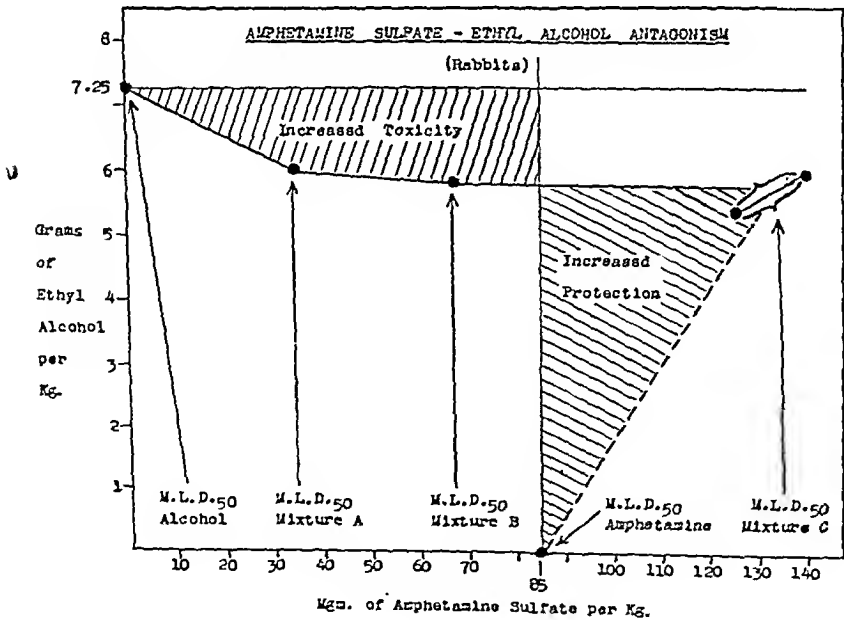


Fig. 6.

In the lethal dose experiments, however, the addition of amphetamine sulfate up to 1.5 times the minimum lethal quantity had no effect in protecting the rabbits from the M.L.D.<sub>50</sub> or more of alcohol. Indeed, the presence of the

amphetamine increased the toxicity so that the animals tolerated less alcohol (Fig. 6). This suggests a clinical danger which is more apparent than real. In acute alcoholic states in man there is present a quantity of alcohol, which rarely, if ever, approaches a lethal amount, because the hypnotic action of alcohol usually manifests itself long before a lethal quantity can be consumed. In these clinical conditions, therefore, the use of amphetamine sulfate is not likely to be attended with the danger of producing increased toxicity.

In contrast to these findings, alcohol up to 80 per cent of a lethal quantity protected the rabbits from 1.5 times the minimum lethal dose of amphetamine sulfate. This increased protection has been confirmed by Werner,<sup>5</sup> previously cited. Because of the widespread employment of amphetamine sulfate in various disease conditions, the possibility of using alcohol as an antidote in states of acute amphetamine poisoning should be kept in mind.

The possibility that these manifestations are due to disturbances in absorption must be considered. However, if the amphetamine interfered with the absorption of the alcohol, there should be an increased protection against alcohol when large doses of alcohol and small doses of amphetamine are used together, instead of an increased toxicity. Likewise, even if the alcohol interfered with the absorption of amphetamine, this would not explain the occurrence of the increased toxicity to alcohol when large doses of alcohol and small doses of amphetamine are employed together.

#### SUMMARY

Four hundred twenty-three rabbits were selected as to age (6 to 15 months), weight (1.70 to 3.00 kg.), sex (equal numbers), and given solutions of amphetamine sulfate and/or ethyl alcohol by stomach tube. The standard laboratory diet was withheld for over sixteen hours before and after administration.

The following data were obtained:

(1) The administration of 5 Gm. per kilogram of alcohol to 13 animals produced narcosis (inability after painful stimulus to hop once without losing balance) with an onset of 17.4 minutes and a duration of 408 minutes (average). (2) The administration of 5 Gm. per kilogram of alcohol with 1 M.L.D.<sub>50</sub> (85 mg.) of amphetamine per kilogram to another 13 animals produced narcosis with an onset of 55.9 minutes and a duration of 307 minutes (average). The addition of amphetamine delayed the onset of the narcosis about 200 per cent and shortened it about 25 per cent.

(3) The M.L.D.<sub>50</sub> of 25 per cent alcohol (38 animals) was 7.25 Gm. per kilogram. (4) The M.L.D.<sub>50</sub> of 25 per cent amphetamine (68 animals) was 85 mg. per kilogram. (5) The M.L.D.<sub>50</sub> of mixture A (1 M.L.D.<sub>50</sub> of alcohol and  $\frac{1}{2}$  M.L.D.<sub>50</sub> of amphetamine per unit volume) (63 animals) was 6.025 Gm. per kilogram of alcohol with 35.32 mg. per kilogram of amphetamine. (6) The M.L.D.<sub>50</sub> of mixture B (1 M.L.D.<sub>50</sub> of alcohol and 1 M.L.D.<sub>50</sub> of amphetamine per unit volume) (55 animals) was 5.78 Gm. per kilogram of alcohol with 67.72 mg. per kilogram of amphetamine. (7) The M.L.D.<sub>50</sub> of mixture C ( $\frac{1}{2}$  M.L.D.<sub>50</sub> of alcohol and 1 M.L.D.<sub>50</sub> of amphetamine per unit volume) (99 animals) was 125 to 140 mg. per kilogram of amphetamine with 5.329 to 5.968 Gm. per kilogram of alcohol.

## CONCLUSIONS

In the rabbit (1) amphetamine sulfate has an antagonistic effect on the narcosis of moderate amounts of ethyl alcohol. (2) It does not have an antagonistic action on the narcosis of lethal doses, and even increases the toxicity of near-lethal quantities. (3) Ethyl alcohol, on the contrary, protects the animal from 1.5 to 2 times the M.L.D.<sub>50</sub> of amphetamine sulfate.

In man these findings may be correlated with the antagonistic effect of amphetamine sulfate in acute alcoholic states. Inasmuch as such conditions involve only moderate quantities of alcohol, and rarely, if ever, near-lethal amounts, the possibility of increased toxicity from the amphetamine sulfate-ethyl alcohol combination is remote.

Alcohol may be of value as an antidote in states of acute amphetamine poisoning.

Grateful acknowledgment is made to Dr. M. S. Dooley for advice and criticism, to Dr. H. P. Smith for statistical analyses of the results, and to Dr. John Rowlingson for technical assistance.

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## TOXIC REACTIONS TO SULFAPYRIDINE\*

ACUTE HEMOLYTIC ANEMIA, LEUCEMOID REACTION, AND PURPURA IN  
THREE SEPARATE CASES

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SULFAPYRIDINE, like most other potent chemotherapeutic agents, has concomitant side effects and toxic reactions. A knowledge of these facts cannot be too strongly stressed for the safe administration of the drug. The deleterious effect of sulfapyridine on hemopoiesis of susceptible individuals is among the most dangerous of its toxic actions. Several cases<sup>1-6</sup> of bone marrow depression, as evidenced by leucopenia with agranulocytosis or thrombocytopenia, have been reported from large or even small doses of the drug. This is not surprising, since the benzene ring is present in sulfapyridine and is capable of causing blood

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reactions affecting the formed elements of the blood either singly or in combination, producing acute hemolytic anemia, unusual leucocytosis, or leucemoid blood pictures, leucopenia with malignant neutropenia, or agranulocytosis and thrombocytopenia with hemorrhagic phenomena.

To date, there has been only one report<sup>7</sup> of leucemoid reaction due to inordinate stimulation of the bone marrow by sulfapyridine. It is, therefore, felt worth while to describe three additional cases of serious bone marrow damage caused by this drug, which produced this leucemoid blood picture in addition to severe hemolytic anemia in the first two patients, and thrombocytopenic purpura in the third patient.

#### CASE REPORTS

CASE 1.—F. C. (No. 105350), a 25-year-old male was admitted on Dec. 25, 1939, and discharged on Jan. 14, 1940. He complained of fever, chills, cough with expectoration of greenish, blood-stained sputum, and pain in the right chest for six days before admission. Physical examination revealed an acutely ill colored male with tachypnea, widened alae nasi, dullness of right upper chest anteriorly, where bronchovesicular breathing and crackling râles were heard. The clinical diagnosis of pneumonia of the right upper lobe was corroborated by x-ray taken the day of admission.

The urine, blood glucose, and nonprotein nitrogen on admission were normal. The Wassermann test was negative.

The patient was given 30 grains of sulfapyridine on admission (Dec. 25, 1939) and 15 grains every four hours until Jan. 1, 1940. The temperature, which was 102° F. on admission, dropped to 99° F. by December 27, and stayed at a normal level until December 31, when it rose to 101° F. for four days. The secondary rise in temperature on December 31, was due to the action of the drug causing a severe hemolytic anemia (Table I) and not to the pneumonic process which showed complete resolution both by physical signs and x-ray on Jan. 1, 1940.

It will be noted that the hemoglobin of 95 per cent on admission fell to 60 per cent after four days of the administration of sulfapyridine, and the red blood cells fell from 4.5 to 3 million. There was a marked leucocytosis (31,000) with the appearance of many premature forms in the circulating blood. By Jan. 1, 1940, the red blood cells had fallen to exceedingly low proportions: the hemoglobin fell to 20 per cent with 1.5 million red blood cells. The white blood count rose to 64,800. The patient now exhibited an icteric tinge of the skin. The icteric index was 10; the van den Bergh was positive and delayed; urobilin excretion was increased in the urine at this time; wet preparation for sickling of erythrocytes was negative. The sulfapyridine was immediately discontinued, and a blood transfusion was given on the same day; this was repeated two days later. A bone marrow puncture revealed a hyperactive erythronormoblastic marrow (Table I). When the temperature fell to normal on January 4, the blood rapidly returned to normal, and by January 8 no abnormal elements were seen in the blood. The patient's clinical condition improved rapidly and he was discharged, fully recovered, on Jan. 14, 1940.

The marked acute hemolytic anemia and the marked response of the granulocytic cells with many immature forms produced a leucemoid blood picture in this case, which lasted only a few days and responded to blood transfusion after discontinuance of sulfapyridine.

CASE 2.—J. T. (No. 106133), a 49-year-old male, was admitted on Jan. 17, 1940, and discharged on Feb. 11, 1940. The patient complained of pain in the left chest, with fever, cough, and expectoration of thick sputum for four days.

Physical examination revealed an acutely ill patient with a temperature of 103° F. There were absolute dullness, bronchophony, bronchial breathing, and crackling râles at the left base of the lung. The clinical diagnosis of left lower lobe pneumonia was corroborated by x-ray on Jan. 17, 1940.

The patient was given 30 grains of sulfapyridine on admission. This was repeated four hours later, and then 15 grains were given every four hours. On January 21 and 22, icterus developed. The blood count (Table II) showed rapidly developing anemia. The sulfapy-

ridine was discontinued on January 22, and blood transfusion was given. This was repeated on January 24 and January 28. As the blood chart shows (Table II), there was a milder leucemoid reaction in this case than in the former one (Table I), the height of the leucocyte rise being only 28,750. Numerous abnormal cells were found in the circulating blood. The hemolytic process also seemed milder than in the first case. Sickling in wet preparations was negative. The bone marrow in this patient (Table II) was similar to the previous case and showed a hyperplastic erythronormoblastic tendency. When the patient was discharged, he had fully recovered from the toxic effects of sulfapyridine on the blood.

CASE 3.—M. K., a 50-year-old female, was admitted on Nov. 23, 1939, and discharged on Jan. 7, 1940. Her illness began on November 18, with pain in the chest, cough, chill, and blood-tinged sputum. A diagnosis of pneumonia was made prior to admission and about 60 grains of sulfapyridine were administered daily. She entered the hospital because of the increase in chest pain and rise in temperature.

Physical examination revealed marked dullness, increased tactile and vocal fremitus, and fine crepitant râles over the right middle and lower lobes. The clinical diagnosis of pneumonia was substantiated by x-ray on November 24, showing clouding in the right side, with suggestion of some interlobar pleural thickening.

The admission temperature was 103° F. Maintenance doses of sulfapyridine (90 grains in all) were administered. The temperature declined to 98° F. on the third day, when bright red blood was expectorated. Two hemorrhagic papillomatous masses were observed on either side of the buccal mucous membrane near Stensen's duct. There were elevated red papillae at the borders of the tongue and hemorrhage in the gingival tissue of the lower jaw. In addition, there was moderate vaginal bleeding. That night a generalized rash was noticed, with hemorrhagic papillomatous masses throughout the buccal mucous membrane. There was also hemorrhage into the left sclera. The sulfapyridine was discontinued, and a blood transfusion was given with 500 c.c. of saline and coagamine (thromboplastin). The following day the patient showed marked pallor. Purpura appeared in scattered patches over the trunk and extremities, as well as a large ecchymotic area over the left thigh. This constituted the height of the toxic reaction. With transfusion, coagamine, and time the patient recovered and was discharged well, except for some chronic pneumonitis and pleural thickening as a residue of her pneumonia.

As a study of the blood shows (Table III), this patient entered the hospital with an anemia (54 per cent hemoglobin, 3,500,000 red blood cells), undoubtedly due to the administration of sulfapyridine. The anemia alone was not considered sufficiently profound to withhold sulfapyridine because of the clinical picture of the patient. The purpuric manifestations which developed on the fourth day were of the secondary type of thrombocytopenic purpura, with low platelet count (25,000), prolonged bleeding time (twenty-five minutes), and delayed clot retraction time. The leucemoid reaction in this patient (35,000 W.B.C. with 12 per cent neutrophilic myelocytes) was less marked than in the first case, but still forms an important part of this patient's toxic reaction to sulfapyridine. The bone marrow study here was not significant, because it was taken after transfusion, and it failed to show depression or hyperactivity (Table III).

#### DISCUSSION

Although the two drugs sulfanilamide and sulfapyridine are closely related, and their toxic effects are quite similar, this discussion is concerned with the latter drug, and shall be limited to its toxic action on the blood. This is probably the most important toxic effect of sulfapyridine, since it occurs not infrequently, is usually severe, and occasionally is fatal. Some degree of anemia is reported in fully 3 per cent of 3,000 cases that received sulfapyridine.<sup>8</sup> The anemia produced by the drug is of the acute hemolytic type, usually occurs during the first three or four days of therapy with rapid hemolysis of the red blood cells, and a rise in serum bilirubin. There is a rapid decline in hemoglobin and red blood cells to alarmingly low levels of 20 per cent, and even

TABLE 1

CASE 1. PERIPHERAL BLOOD

| DATE  | Hb.<br>% | R.B.C. | W.B.C. | NEU-<br>TRO-<br>MYEL. | META-<br>MYEL. | NON-<br>SEG. | SEG-<br>MENT | LYM. | MONO. | EOS. | BASO. | RETIC.<br>% | NORMO-<br>BLASTS<br>100<br>W.B.C. | RED BLOOD CELL MORPHOLOGY   |
|-------|----------|--------|--------|-----------------------|----------------|--------------|--------------|------|-------|------|-------|-------------|-----------------------------------|---|
| 12/25 | 95       | 4,50   | 17,0   |                       |                | 18           | 59           | 20   | 3     |      |       |             |                                   |   |
| 12/29 | 60       | 3,10   | 31,0   | 8                     |                | 38           | 27           | 13   | 14    |      |       |             |                                   |   |
| 1/1   | 20       | 1,50   | 64,8   | 3                     | 8              | 35           | 45           | 8    | 1     |      |       |             | 15                                | Anisocytosis, poikilocytosis, hypo-<br>chromin, polychromatophilic  |
| 1/2   |          |        | 60,0   | 1                     | 12             | 35           | 45           | 6    |       |      | 1     | 16.4        | 23                                | Anisocytosis, poikilocytosis, macrocy-<br>tosis, polychromatophilic |
| 1/3   | 44       | 1,65   | 46,2   |                       | 15             | 20           | 51           | 9    | 5     |      |       |             |                                   | Anisocytosis, poikilocytosis, macrocy-<br>tosis, polychromatophilic |
| 1/5   | 60       | 2,00   | 7,25   |                       | 2              | 11           | 59           | 20   | 8     |      | 1     | 25.6        | 7                                 | Anisocytosis, poikilocytosis, macrocy-<br>tosis, polychromatophilic |
| 1/8   | 70       | 3,15   | 6,00   |                       |                | 5            | 65           | 23   | 3     | 3    |       | 7.2         | Rare                              | Anisocytosis, poikilocytosis, macrocy-<br>tosis, polychromatophilic |
| 1/11  |          |        |        |                       |                | 1            | 60           | 34   | 3     | 1    | 1     | 3.2         | Rare                              | Anisocytosis, poikilocytosis, macrocy-<br>tosis, polychromatophilic |

CASE 1. BONE MARROW IN THE ANEMIC PHASE

| DATE        | TOTAL<br>COUNT | MYELO-<br>BLASTS | PRO-<br>MYEL. | NEU-<br>TRO-<br>MYEL. | EOS.<br>MYEL. | META-<br>MYEL. | NON-<br>SEG. | SEG-<br>MENT | LYM. | MONO. | EOS. | PLASMA<br>CELL | MIGRA-<br>BLASTS<br>(PER 100 W.B.C.) | ERYTHRO-<br>BLASTS | NORMO-<br>BLASTS | REMARKS           |
|-------------|----------------|------------------|---------------|-----------------------|---------------|----------------|--------------|--------------|------|-------|------|----------------|--------------------------------------|--------------------|------------------|-------------------|
| 1940<br>1/2 | 542,000        |                  |               | 5                     | 2             | 24             | 30           | 31           | 3    |       | 2    |                | 6                                    | 79                 | 185              | Hyper-<br>plastic |

In Recovery Phase

|      |      |  |   |  |  |    |    |    |    |   |   |  |   |    |  |    |
|------|------|--|---|--|--|----|----|----|----|---|---|--|---|----|--|----|
| 1/11 | 64,8 |  | 2 |  |  | 15 | 17 | 36 | 15 | 2 | 5 |  | 2 | 12 |  | 62 |
|------|------|--|---|--|--|----|----|----|----|---|---|--|---|----|--|----|

TABLE II  
CASE 2. PERIPHERAL BLOOD

| DATE | Hb. % | R.B.C. | W.B.C. | NEU-<br>TRO-<br>MYEL. | META-<br>MYEL. | NON-<br>SEG. | SEG-<br>MENT | LYM. | MONO. | EOS. | BASO. | RETIC.<br>% | NORMO-<br>BLASTS<br>100<br>W.B.C. | RED BLOOD CELL MORPHOLOGY  |
|------|-------|--------|--------|-----------------------|----------------|--------------|--------------|------|-------|------|-------|-------------|-----------------------------------|--|
| 1/17 | 72    | 3,51   | 11,2   | 2                     | 11             | 33           | 58           | 9    | 6     | 3    |       |             | 7                                 | Anisocytosis, Poikilocytosis<br>Anisocytosis, Poikilocytosis<br><br>Anisocytosis, Poikilocytosis |
| 1/22 | 32    | 1,81   | 25,4   |                       | 7              | 21           | 49           | 8    |       |      |       | 4           | 7                                 |  |
| 1/23 | 30    | 1,11   | 25,0   | 1                     | 7              | 28           | 53           | 4    | 7     | 2    |       | 9.4         | 2                                 |  |
| 1/24 | 32    | 1,70   | 28,7   | 1                     | 5              | 14           | 63           | 11   | 4     |      |       | 13.4        |                                   |  |
| 1/25 | 43    | 1,76   | 17,15  |                       |                | 13           | 74           | 9    | 4     |      | 2     | 8.4         | 8                                 |  |
| 1/29 | 58    | 2,69   | 11,15  |                       |                |              | 66           | 24   | 8     | 2    |       | 0.6         | 9                                 |  |
| 1/31 | 60    | 2,81   | 12,12  |                       |                |              | 65           | 24   | 9     | 2    | 1     | 0.5         | 10                                |  |
| 2/4  | 74    | 3,43   | 9,55   |                       |                | 1            | 30           | 56   |       |      |       |             |                                   |  |
| 2/8  |       |        |        |                       |                |              |              |      |       |      |       |             |                                   |  |

CASE 2. BONE MARROW IN THE ANEMIC PHASE

| DATE                     | TOTAL<br>COUNT | MYELO-<br>BLASTS | PRO-<br>MYEL. | NEU-<br>TRO-<br>MYEL. | EOS.<br>MYEL. | META.<br>MYEL. | NON-<br>SEG. | SEG-<br>MENT | LYM. | MONO. | EOS. | PLASMA<br>CELL | MEGALO-ERYTHRO-<br>BLASTS (PER 100 W.B.C.) | NORMO-<br>BLASTS | REMARKS           |
|--------------------------|----------------|------------------|---------------|-----------------------|---------------|----------------|--------------|--------------|------|-------|------|----------------|--|------------------|-------------------|
| 1/40                     |                |                  | 2             | 11                    | 2             | 20             | 35           | 21           | 5    |       | 2    | 2              | 7  | 32               | Hyper-<br>plastic |
| 1/23                     |                |                  |               |                       |               |                |              |              |      |       |      |                |  | 111              |                   |
| <i>In Recovery Phase</i> |                |                  |               |                       |               |                |              |              |      |       |      |                |  |                  |                   |
| 2/8                      | 66,8           | 1                | 3             | 12                    | 2             | 22             | 24           | 17           | 12   | 1     | 5    |                | 2  | 37               |                   |

TABLE III  
CASE 3. PERIPHERAL BLOOD

| DATE        | Hb. | R.B.C. | W.B.C. | PLATE-<br>LETS | NEUTRO-<br>MYEL. | META-<br>MYEL. | NON-<br>SEG. | SEG-<br>MENT | LYM. | MONO. | EOS. | BASO. | RETIC.<br>% | NORMO-<br>BLASTS<br>100<br>W.B.C. | RED BLOOD CELL<br>MORPHOLOGY           |
|-------------|-----|--------|--------|----------------|------------------|----------------|--------------|--------------|------|-------|------|-------|-------------|-----------------------------------|--|
| 10/30       |     |        |        |                |                  |                |              |              |      |       |      |       |             |                                   |  |
| 11/27       | 54  | 3,50   | 23,8   | 25,000         | 2                |                | 22           | 63           | 11   | 1     | 1    |       |             |                                   |  |
| 11/27       | 54  | 3,37   | 28,3   | 25,000         | 12               |                | 25           | 33           | 11   | 2     | 2    |       |             |                                   |  |
| 11/29       | 44  | 2,80   | 33,0   |                |                  | 15             |              |              |      |       |      |       |             |                                   |  |
| 12/1        | 43  | 2,40   | 19,5   |                | 8                |                | 26           | 30           | 15   | 6     | 5    |       |             | 1                                 | Slight anisocytosis,<br>poikilocytosis |
| 12/5        | 63  | 3,24   | 11,1   | 355,000        | 3                |                | 14           | 42           | 26   | 11    | 3    |       |             |                                   |  |
| 12/8        | 63  | 3,44   | 9,8    | 250,000        | 1                |                | 7            | 62           | 28   | 2     |      |       |             |                                   |  |
| 12/11       | 62  | 3,09   | 8,1    | 325,000        | 3                |                | 10           | 48           | 30   | 8     | 1    |       |             |                                   |  |
| 12/16       | 62  | 3,80   | 9,7    |                |                  |                | 10           | 54           | 32   | 4     |      |       |             |                                   |  |
| 12/20       | 66  | 3,60   | 6,3    | 250,000        |                  |                | 7            | 52           | 33   | 6     | 2    |       |             |                                   |  |
| 1940<br>1/2 | 75  | 3,30   | 7,2    |                |                  |                | 3            | 45           | 52   |       |      |       |             |                                   |  |

CASE 3. BONE MARROW

| DATE  | TOTAL<br>COUNT | MYELO-<br>BLASTS | PRO-<br>MYEL. | NEU-<br>THO-<br>MYEL. | EOS.<br>MYEL. | META-<br>MYEL. | NON-<br>SEG. | SEG-<br>MENT | LYM. | MONO. | EOS. | PLASMA<br>CELL | MEGALO-ERYTHRO- BLASTS     |        | REMARKS  |
|-------|----------------|------------------|---------------|-----------------------|---------------|----------------|--------------|--------------|------|-------|------|----------------|----------------------------|--------|--|
|       |                |                  |               |                       |               |                |              |              |      |       |      |                | BLASTS<br>(PER 100 W.B.C.) | BLASTS |  |
| 10/30 |                |                  |               |                       |               |                |              |              |      |       |      |                |                            |        |  |
| 12/2  |                |                  | 3             | 4                     |               | 23             | 22           | 34           | 10   |       | 4    |                | 1                          | 20     | Occasional<br>mega-<br>karyo-<br>cytes;<br>very nu-<br>merous<br>platelets |



to one million red blood cells with all the findings of an acute hemolytic crisis, with the presence of frequent normoblasts, anisocytosis, poikilocytosis, frequent macrocytosis, hyperchromia, polychromatophilia, basophilic stippling, and reticulocytosis (Fig. 1). The peak of the reticulocytosis occurs at the third or fourth day; in Case 1, to a rise of 25.6 per cent, and in Case 2, to 13.4 per cent. The action of the drug appears to be entirely peripheral, because the few bone marrow studies, including our own, which have been made in these cases, show no depression but rather hyperplasia and increased hematopoietic activity of the marrow. The bone marrow in the first two cases (Tables I, II) showed great cellularity and hyperplasia, especially of the erythropoietic elements, such as an increase in erythroblasts, with normoblasts and occasional megaloblasts (Figs. 2 and 3). This hyperplasia is undoubtedly due to an attempt on the part of

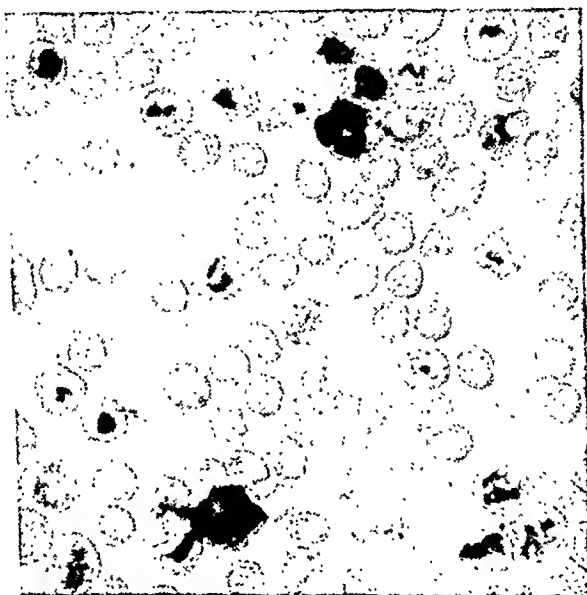


Fig. 1.—Showing reticulocytosis.

the bone marrow to compensate for the rapid intravascular blood destruction in an effort to meet the increasing demands for regeneration in order to combat the anemia. The myeloid elements show a moderate degree of hyperplasia with a shift to the left. Although the hemolysis and anemia are somewhat progressive even after the withdrawal of sulfapyridine, the anemia usually improves with repeated blood transfusions. It is of interest to note that, with clinical improvement showing an increase in hemoglobin and red blood cells, the bone marrow shows a corresponding reduction in erythroid cells.

Since there is no abnormal fragility of the red blood cells in these cases, it may be assumed that they are destroyed directly by some hemolytic process or indirectly through the reticulo-endothelial system. It is known that the effect of sulfapyridine on the blood is often selective, causing hemolytic anemia with moderate leucocytosis. As with sulfanilamide, the most commonly reported damage caused by sulfapyridine is marked reduction of the number of leucocytes with granulocytopenia.<sup>3</sup> The mechanism is probably on a basis of maturation

arrest or hypoplasia of the granulocytic series in the bone marrow, similar to that seen following benzol, aminopyrine, etc. There has been only one report to date of the opposite toxic effects producing a leucemoid picture. Leucemoid reactions have been reported by Heek and Hall<sup>9</sup> and have been observed in overwhelming infections more commonly of coccal than bacillary origin, sepsis, uremia, occasional heavy metal poisons, measles, infectious mononucleosis, hemolytic anemia, polycythemia vera, instances of the lymphomatous diseases, Albers-Schönberg disease, and multiple myeloma. The leucocytosis observed resulting from sulfanilamide or sulfapyridine may be due to actual irritation of the bone marrow rather than to the underlying infection, because it often occurs when

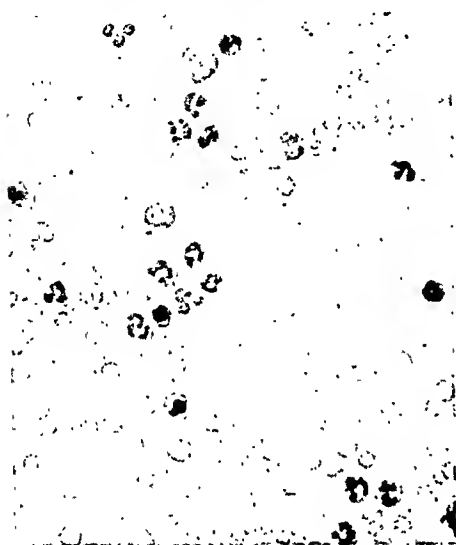


Fig. 2.

Fig. 2.—Peripheral blood smear showing marked leucocytosis with few myelocytes and normoblasts.

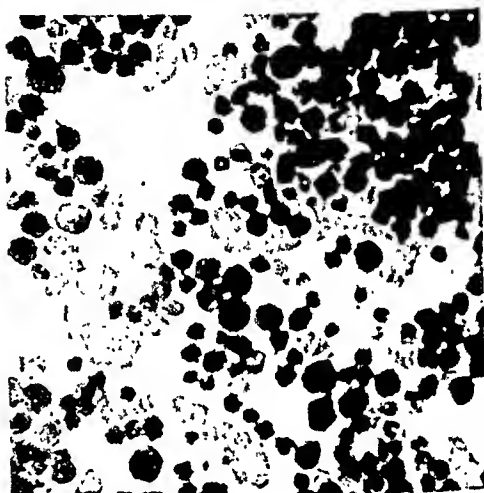


Fig. 3.

Fig. 3.—Hyperplastic erythronormoblastic bone marrow showing numerous erythroblasts, normoblasts, and myeloid elements.

the infection is on the decline. A diagnosis of leucemia is sometimes ventured, but the presence of a potent producing cause, the dramatic clinical improvement of the patient, and the rapid return of the blood to normal should offer no difficulties in excluding leucosis. The case reported by Moody and Knouf<sup>7</sup> is similar to those reported here, showing the rather common acute hemolytic anemia and the very uncommon leucemoid reaction which responds satisfactorily after blood transfusion.

The occurrence of secondary thrombocytopenic purpura with a mild leucemoid reaction, reported in our third case, raises the question of the possible allergic reaction of sulfapyridine on the blood. According to the allergic theory,<sup>10</sup> drugs like sulfanilamide and sulfapyridine produce thrombocytopenic purpura by sequestration or destruction of platelets caused by widespread alteration of the reticulo-endothelial system in persons who have a constitutional predisposition with acquired sensitivity to certain drugs. Although the theory cannot be easily disproved, Hodes and associates<sup>3</sup> offer good evidence that so far as the leucopenia is concerned, the specific hematologic reaction patterns

are not due to acquired sensitivity, because patients who developed leucopenia in their first course of treatment with sulfapyridine failed to show similar reaction during their second course of treatment.

It must be admitted that as yet the exact mechanism in which sulfapyridine affects the blood and blood-forming organs adversely is not known. Nevertheless, we cannot help feeling strongly that a complete blood count should be done to determine the condition of the patient's blood before administration of the drug. Repeated blood counts either daily or at least every other day must be done so that toxic effects on the hemoglobin, red blood cells, leucocytes, and platelets may be readily detected, the drug discontinued in time, and repeated blood transfusion administered to avoid fatal outcome in these unfortunate cases.

### CONCLUSIONS

Unusual toxic reactions of the blood to sulfapyridine are reported in three cases with acute hemolytic anemia, and varying grades of leucemoid blood pictures and associated secondary thrombocytopenic purpura. In spite of these serious reactions all the cases recovered in a short time after transfusions. Bone marrow studies showed erythroblastic hyperactivity in the two cases. The theories of the effects of the drug on hemopoiesis are discussed. It is believed that the marked hemolytic anemia may be evidence of a peripheral action; the leucocytosis an irritation of the myeloid element of the bone marrow; and the thrombocytopenic purpura an allergic phenomenon. The importance of frequent blood counts on any patient receiving sulfapyridine cannot be overemphasized.

We wish to thank Professor Linn J. Boyd for the privilege of reporting these cases.

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## PLEURAL SHOCK—A REFLEX\*

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### PRELIMINARY REPORT

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FOR many years the etiology of pleural shock has been debated; men of eminence have suggested that the cause is air embolus, while others of equal importance believe the condition is reflex in origin.

Studies have been made in which blood pressure, pulse rate, and respiration have been carefully checked. These merely serve to confirm the existence of pleural shock, but do not demonstrate its etiology.

It has been the custom here in the surgical laboratory to sacrifice animals by injecting ether into the chest cavity. Almost immediately after the injection the animal has a mild convulsion, rolls on its back, and within two or three minutes is dead, respirations having become rapid and shallow, and finally ceasing; heart action also stopping after what seems like a short period of increase in activity. Pupils become dilated; cyanosis is marked.

It occurred to us that the time elapsed was too short to account for the death on other than a reflex basis, and that the ether could not have caused an anesthetic death in so short a time. We postulated a vagovagal reflex (pleurocardiac) and consulted the literature for support. The reflex was suggested as early as 1865 and has been tentatively confirmed by experimental work in which atropine prevented or diminished signs of pleural shock, as did vagotomy.

We, therefore, sacrificed several rabbits left from other work by injecting ether in the pleural cavity, and taking electrocardiograms throughout the procedure. We have succeeded in killing rabbits with as little as 0.5 c.c. of ether in as little as one minute. Autopsies have revealed no gross evidence of air embolism.

Manometer readings are taken to show that the ether has been injected into the pleural cavity. Pressure increases are relatively small, showing that cardiac embarrassment due to mediastinal shift is not responsible for the deaths.

Electrocardiographic tracings are taken in all three limb leads before insertion of needle into chest. Lead II is then taken after insertion of needle and during injection of ether, and until after clinical death is evident. The records show changes in the T-wave, inversion and flattening being pronounced, and the take-off high.

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We expect to continue this work on rabbits and larger laboratory animals. Tracings will also be made on human beings whose chests are entered for thoracentesis, pneumothorax, and surgery.

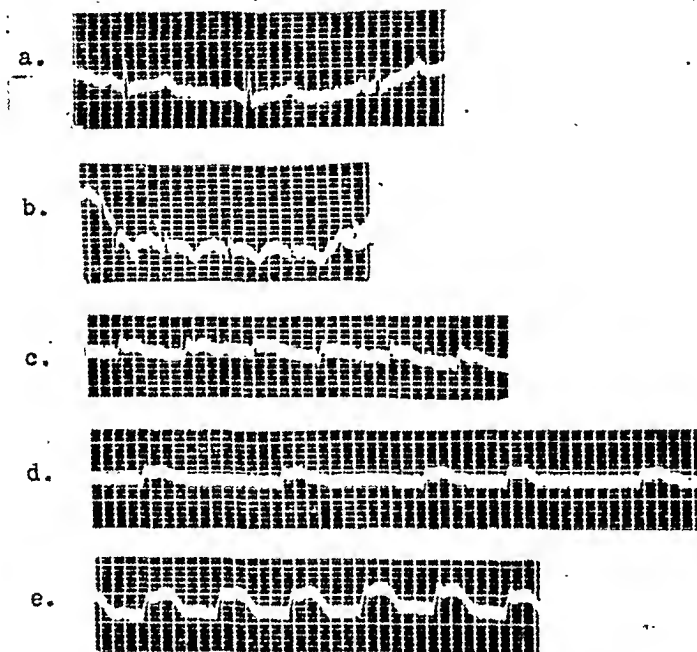


Fig. 1.—*a.* Normal electrocardiogram of a rabbit; P-waves are positive. Initial complex shows low R-wave neither split nor widened. S-T segment is in isoelectric line. T-waves are positive. *b.* Taken 67 seconds after injection, shows tachycardia, short Q-wave. R-wave is lower, T-wave is inverted. *c.* Taken 80 seconds after injection, shows high take-off of S-T segment and T-wave from the descending part of R-wave. *d.* Taken 89 seconds after injection, shows arrhythmia. P-waves are not distinctly visible. Ventricular complex is unaltered. *e.* Taken 131 seconds after injection, shows regular tachycardia with marked high take-off, causing almost a monophasic electrocardiograph.

This report is submitted so that others, dealing mainly with chest pathology and with opportunity to apply this investigation to human beings, may check our early impressions with actual clinical experience.

## ENDOCRINE ASPECTS OF HEADACHES

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THE problem of headaches has been investigated from several viewpoints in accordance with the fact that pain may be caused by a variety of pathologic processes affecting the structures of the head, as well as by a variety of systemic conditions, both infectious and metabolic. Headaches also develop in the absence of all organic disease as an expression of disturbances in the emotional sphere or of a psychoneurotic state. However, those either of the migraine, or a less specific type, frequently occur in patients who do not present any, or not enough, organic pathology to account for their complaints; nor can the headaches be blamed on nervous or mental conditions.

The occurrence of headaches in patients who consult the endocrinologist for varied reasons is so common that it seemed justified to investigate the possible causal relationship between endocrinopathies and attendant metabolic disorders on the one hand, and the attacks of headaches on the other.

A series of 50 consecutive cases of severe headaches is presented, most of which were of the characteristic migraine type. All cases, including those clinically not classified as migraine, were noteworthy for their severity, frequency, and refractoriness to ordinary palliative treatment. Organic pathology or systemic disease as the cause of these headaches could be ruled out by thorough clinical examination.

Oliguria was a distinct feature, for the twenty-four-hour urine output was less than 1,000 c.c. in the majority of these cases. The daily urinary volume was above that figure in one-third of the group, with a maximum of 1,780 c.c. in one patient; but all these patients admitted an increased liquid intake. The twenty-four-hour urine specimens showed an average sodium chloride content of only 6.7 Gm. in spite of the fact that a good majority of the patients admitted an increased liking for, and consumption of, salty food.

A salt tolerance test was performed on all patients. It consisted of the administration of 10 Gm. of sodium chloride and 250 c.c. of water in addition to the identical food and liquid intake of the preceding control day. There was a rise of the twenty-four-hour salt excretion to 9.4 Gm. as an average, indicative of a retention of 73 per cent of the test salt for the whole group. Only 6 persons showed complete elimination of the added 10 Gm. of salt. On the other hand, complete retention of the test salt was noted in a similar number of cases, while the chloride excretion after salt intake was actually smaller than on the control day in 8 persons.

The twenty-four-hour urinary volume averaged 1,040 c.c. for the whole series; it decreased to 947 c.c. on the test day in spite of the fact that the liquid intake was 250 c.c. higher. In other words, the average retention was 343 c.c. It might be mentioned that water was retained in only one of the 6 patients who did not retain salt, whereas the other 5 eliminated increased amounts of urine. Thirty-two patients, i.e., a majority of the group, showed an actual decrease in urine output, and a slight increase, but less than the 250 c.c. of additional liquid intake, was noticeable in the remaining 13.

Estimation of blood chlorides carried out on whole blood was not found enlightening until the study was extended to the examination of chlorides separately in red blood cells and plasma.

The values for chlorides in the whole blood were found within the normal range from 480 to 510 mg. per cent. Higher values up to 530 mg. per cent were obtained only in 10 persons. The sodium chloride content of the red blood cells, however, ranged from 390 to 430 mg. per cent, exceeding the upper limit of our normal figures (380 mg. per cent) in 32 cases, that is, in 64 per cent of the series.

Studies of the sodium content of the blood were carried out only on the last 10 patients of this group. The values found varied between 230 and 264 mg. per cent, whereas control figures obtained with the same methods reveal the normal sodium level at 200 to 220 mg. per cent.

Determination of the basal metabolism showed that the average rate for the group was minus 10; the individual figures varied from minus 27 to plus 13, with the majority of the group well below minus 10. The specific dynamic action of proteins determined two hours after the intake of a small protein meal revealed an average increase of the metabolic rate of only 5 points. No rise at all was noted in almost one-half of the group; a considerable increase of the specific dynamic action of 23 to 28 points was observed in 6 persons. Upon elimination of these few patients who seem to deserve special consideration, the remaining 44 showed an average specific dynamic action as low as 2 points.

The fasting blood sugar ranged from 65 to 108 mg. per cent, with an average of 85 mg. per cent which is close to the lower limit of our normal range. Uric acid values were found to be from 2.2 to 4.8 mg. per cent, with an average of 3.4 mg. per cent which is definitely above normal. The differential count of the white blood cells showed variations of the lymphocyte count from 12 to 55 per cent, yet the average was 36 per cent, definitely connoting a relative lymphocytosis.

#### DISCUSSION

Our study seems to show the definite tendency in our group of patients to produce a low volume of urine with reduced sodium chloride output. There is marked retention of salt during the performance of the salt tolerance test, accompanied by water retention, which in some of the cases became visible in the form of swellings or seemed to precipitate a migraine spell. Clinically, two facts deserve emphasis: (1) the frequency of demonstrable vasomotor disturbances, such as white dermographism, together with a variety of related clinical manifestations, such as urticaria, Quinke's edema, rhinitis, and "sinus trouble"; (2) the prevalence of endocrine stigmatization. The latter includes,

first of all, the physical appearance of the patients who seem to fall mainly into two groups. One group included patients of both sexes who were tall, lean, and of slightly or definitely eunuchoidal proportions, while most of the patients of the other group showed peripelvic fat deposits such as are thought to be characteristic for pituitary insufficiency. The pattern or changes described as characteristic for a disturbed state of pituitary function (Goldzieher) conforms with the trend demonstrable in this group: low basal metabolism, decreased specific dynamic action of proteins, relative lymphocytosis, increased uric acid, and low normal fasting blood sugar.

That migraine is likely to occur in individuals, earmarked by their physical appearance as being affected with a disturbance of pituitary function, was stressed long ago by Timme, who suggested that the migraine attack might be precipitated by pressure of an enlarged pituitary upon the intracranial tissues. Such an enlargement might be the result of an increased blood and lymph flow to this gland, similar to the directly observable change in volume of the thyroid. Timme deserves credit for having pointed out the frequent coincidence of pituitary stigmatization and habitual headaches in association with angioneurotic manifestations, such as urticaria, hay fever, or asthma. Yet his explanation of increased intracranial pressure as the result of swelling and temporary enlargement of the pituitary is not tenable. X-ray studies show that the sella turcica was, in two of three cases, either enclosed by overlapping clinoid processes, or its aperture was narrowed to such an extent that bulging of the pituitary into the cranial cavity does not seem to be conceivable. An enlarged sella with an aperture wide enough to permit the pituitary to bulge into the cranial cavity was encountered only exceptionally in our series.

The relationship of the pituitary to intracranial pressure, and the causation of headaches, has aroused renewed interest through the findings of Riley, Brickner, and Kurzrok, who demonstrated the appearance of the pituitary gonadotropic hormone in the urine, generally from one to six days before the onset of a migraine spell. The hormone disappeared on the day of the attack, and occasionally appeared without being followed by headache. The excretion of the gonadotropic hormone was practically continuous in two patients with status migrainicus.

The findings of Riley and co-workers gained added significance through the studies of Kraus, who showed that increased intracranial pressure, regardless of its cause, stimulates the pituitary gland and causes hypertrophy of the anterior lobe. This hypertrophy is the anatomic expression of functional overactivity, demonstrated directly by the appearance of the gonadotropic hormone in the urine, and indirectly by the development of follicular cysts of the ovaries (Kraus).

The findings of Kraus permit the reinterpretation of the observations of Riley and associates in the sense that the increased excretion of gonadotropic hormone in migraine cases is the consequence of the increased intracranial pressure that precedes and eventually elicits the migraine attack. That increased intracranial pressure is capable of causing pain needs no argument. As a mat-



ter of fact, pressure or pull on the dura mater, and particularly the dural blood vessels, is considered to be the cause of most headaches (Pickering, Penfield, and Me Naughton).

The mechanism of these functional headaches can be summarized as follows: Due to intracellular retention of salt, and especially of sodium, there is greater avidity of the tissues for water; the attack ensues if increased permeability of the capillaries, as a result of abnormal irritability of the vasomotors, permits a greater flow of moisture to the tissues. The retention of water and subsequent swelling of the tissues within the rigid cranium causes pain which lasts until the water is released through some process of adaptation in which the pituitary is again concerned in some way. The observation that termination of a migraine attack is often accompanied by polyuria is in accord with this view. As further corroboration, we should add that such headaches are quite common in women in connection with the menstrual period; they set in just preceding the menstruation, at a time when water retention is a physiologic event. These menstrual headaches are the best examples for the prevalence and combination of the two responsible factors: water retention and vasomotor instability. The former is proved by the premenstrual gain and postmenstrual loss in weight, while the increased capillary permeability of the premenstrual period is actually demonstrated by the "blister technique" on the skin of normally menstruating women (Peterson and Miller).

In contradistinction to the mechanism of these headaches, which seems to be clarified, the underlying pathology remains an open question. A constitutional, and often familial, element appears to be one of the factors which includes instability of the vegetative nervous system, particularly in respect to the vasomotors. Environmental factors, such as emotional upsets, nervous strain and fatigue, allergic phenomena, or endocrine disturbances are likely to aggravate the vasomotor neurosis. The influence of hormones upon the function of the capillary system is demonstrable experimentally, and clearly shown by clinical observation on menopausal women or castrates. Their outstanding complaints are referable to vasomotor disturbances, which respond well to estrogens, just as the flushes and allied symptoms occasionally noted in the middle-aged male, are relieved by administration of the male sex hormone.

Vasomotor disturbances alone do not cause headaches unless they are associated with a hydropexic state. The retention of water might be the result of long-continued, adverse nutritional habits, such as the excessive intake of salt, polydipsia, a diet low in proteins and high in carbohydrates with vitamin deficiency, as an added possibility. Barring extreme dietary indiscretions, however, it is unlikely that the normal human body, characterized by its power of adaptation, should be unable to take care of an overabundance of water or of food regardless of how unsuitable its composition. Hence, we must postulate a breakdown in the physiologic regulation of salt and water metabolism, such as that obtained in various endocrinopathies. The prevalence of endocrine stigmatization in patients suffering from headaches fulfills this postulate but does not answer the question as to the nature of the endocrinopathy involved, nor as to the hormonal factors that upset salt and water metabolism.

Deficiency of thyroid function may be one of the causes. It is well known that the myxedematous patient retains water and promptly responds to administration of thyroid extract with increased diuresis. Evidence of hypothyroidism, such as short stature, shortness of extremities, firm and dry integument, low basal metabolism, and elevated blood cholesterol, is obtained in quite a few, but certainly not in the majority, of the patients with endocrine headache.

The significance of the adrenal cortical hormone in respect to salt and water metabolism is shown both in the adrenalectomized animal and the patient with Addison's disease who are unable to retain sodium chloride and water normally. Correspondingly, administration of desoxycorticosterone in excessive quantities causes salt and water retention with increased blood pressure and visible edema. Clinically, however, there is no evidence of abnormal adrenal function in the headache patients, hence it would seem that the cortical hormone has no direct bearing upon our problem.

The opposite can be said in respect to the pituitary. Evidence of disordered function of the pituitary was our most common clinical finding, quite in accordance with Timme's observations. Pituitary hormonal factors of known metabolic activity include both those of the anterior and posterior lobes. The anti-diuretic hormone of the posterior lobe is supposed to act chiefly by interference with renal secretion. Important evidence, however, points also to effects directly upon the water content of the peripheral tissues. Experiments on frogs injected with posterior lobe extracts and submerged in water have shown that these animals gain weight by direct absorption and retention of water independently from the function of their kidneys (Brumm). Increased water content of the tissues following injection of posterior lobe extract was actually demonstrated by several investigators (Hines and co-workers, Heller and Smirk). It is significant, moreover, that the antidiuretic effect of the pituitary extract is soon followed by increased diuresis and elimination of chlorides. This justifies the assumption that tissue cells react to the posterior pituitary hormone, first with increased hydration, and subsequently after the effect of the hormone has worn off, with release of water and intracellularly stored sodium chloride.

Anterior lobe extracts have been shown to be diuretic (Teel); it was thought that this effect is referable to the thyrotropic hormone. It has been shown, however, that anterior lobe diuresis is independent of the thyrotropic factor (Mahoney and Sheehan) and is related to metabolic phenomena of a more complex nature (Richter). At any rate, the diuretic effects of the anterior lobe explain the apparent antagonism of the two lobes of the pituitary in respect to diabetes insipidus, for this syndrome develops with impairment of the posterior lobe, but only in the presence of functionally active anterior lobe tissue (v. Hann).

The activity of the anterior lobe enters consideration also in view of its gonadotropic secretion, which is the prerequisite of normal gonad function. The significance of estrogens for water metabolism has been pointed out in recent years by experiments in animals and women (Thorn and associates). The water retention produced by estrogens is in agreement with the fact that women retain water and gain weight at a time when the blood estrogen reaches its peak (At-

kinson and Ivy). Oliguria and refractoriness to diuretic procedures at this time are prevalent (Heilig). This is in striking contrast to the loss of weight and noticeable polyuria with increased elimination of sodium chloride (Thorn and associates) at the time of the postmenstrual low estrogen phase. Swelling of the face, extremities, or other parts of the body, common in the course of the premenstrual retention phase, is likely to subside promptly with the onset of the period (Swecney).

Among the hydrating factors of hormonal nature, insulin must be mentioned, the injection of which causes water retention and may lead to visible edema. While there is no clinical evidence in our group of patients to inculcate the pancreatic islands and to assume an excess of insulin production, it would seem that the hydrating effects of insulin have considerable bearing upon the dietary management of the patient with headache. A diet rich in carbohydrates, especially in those directly convertible into glucose, is likely to stimulate insulin secretion and thus favor retention of water.

#### TREATMENT

Arguments "ex juvantibus" are usually dismissed as complacent and uncritical. If therapeutic procedures, however, are instituted for the correction of demonstrable metabolic abnormalities, and accomplishment of this task is associated with favorable clinical results, the therapeutic test stands up as additional evidence and should not be ignored. In the whole group of 50 patients, there was only one, a highly neurotic woman, who showed little improvement. It is significant that this patient was one of the few who showed no water retention and only a moderate (40 per cent) retention of the test salt. Forty patients, that is, 80 per cent, were completely and permanently relieved by treatment, while 9 showed considerable improvement both as to frequency and severity of their headaches.

This so successful treatment consisted of three elements: diet, medication, and endocrine therapy. The principles of the diet are largely the same as those outlined by Foldes; restriction of salt and liquid intake, high protein content with emphasis on purine-rich meats (liver, sweetbread, sardines), and considerable reduction in the consumption of carbohydrates. Fats are limited in the overweight, but are liberally given in the underweight patient.

The medication is based on two principles: mobilization and increased elimination of sodium, and correction of the imbalance of the vegetative nervous system. Ammonium chloride or potassium acetate given intermittently accomplishes the former and should be applied in the first two or three weeks of the treatment. Atropine combined with phenobarbital or preparations like belladonna are apt to accomplish the second part of our task. In patients with increased blood pressure, the use of bellergal seems to be preferable.

Endocrine medication consists of thyroid and pituitary extracts. It is fallacious to gauge the dosage of thyroid by the basal metabolic rate of the patient. The actual needs are best determined empirically, starting with 0.5 grain daily and gradually increasing the dosage to not more than 2, or at the utmost 3, grains daily. The patient's pulse rate is the best guide for thyroid

medication; slowing down of the pulse rate calls for increased, and undue acceleration for decreased, thyroid dosage. Injection of posterior pituitary extract is of great value, for increased diuresis and salt elimination follow after the initial antidiuretic effect has worn off. The injections should be properly spaced and given not more often than twice a week. The initial dose is 3 minims, with increments of one minim each time until mild abdominal cramps indicate that tolerance has been exceeded. These injections are given preferably in combination with a fresh and potent anterior lobe extract, especially in the presence of menstrual disturbances or other evidence of pituitary deficiency.

This treatment should be kept up for a period of about three months. Subsequently, both injections and medication are discontinued without the danger of recurrence, provided the spirit of the dietary management is kept alive. In other words, these patients must limit permanently the intake of salt and water and adhere to a high protein, low carbohydrate diet. Rigid dieting is not necessary, but overindulgence either in carbohydrates or in liquid consumption was responsible for relapses in several of our patients. Such recurrences promptly yielded to renewed enforcement of the original diet.

#### SUMMARY

A series of 50 cases of headaches, observed in endocrine patients, is presented, and evidence is submitted to show a general trend of sodium chloride and water retention. Stigmata pointing to an endocrine background were prevalent; they include: lower basal metabolism, decreased specific dynamic action of proteins, relative lymphocytosis, higher values for uric acid, sodium and chlorides in the blood, lower blood sugar, and abnormalities in the configuration of the sella turcica.

Endocrine headaches, including migraine headaches, are explained as the result of increased intracranial pressure. They develop when increased permeability of the capillaries permits increased flow of water to, and subsequent retention in, tissues which have stored abnormal quantities of sodium salts.

The retentional type of endocrine headache can be recognized by the decreased output of sodium chloride and water on performing the salt tolerance test.

The mechanism by means of which endocrine factors cause increased hydration of tissues is discussed.

The successful treatment of endocrine headaches consists of a high protein diet with restriction of salt, liquid, and carbohydrate intake, of medication with ammonium or potassium salts and belladonna preparations, and of endocrine substitution therapy with thyroid and pituitary extracts.

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## THE INFLUENCE OF TIME AND VOLUME FACTORS ON VENOUS PRESSURE RESPONSES TO PHYSIOLOGIC SALINE INJECTED INTRAVENOUSLY\*

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COHNHEIM and Lichtheim<sup>1</sup> demonstrated that a 0.06 per cent saline solution would elevate venous pressures without markedly affecting the arterial blood pressure. This finding was corroborated by subsequent investigators,<sup>2-6</sup> who discovered that the systolic output and the size of the heart in diastole were dependent on the elevation of the venous pressure. Caughey<sup>8</sup> suggested that venous pressure responses to intravenous saline be adopted as a test of cardiac function in human subjects, for which purpose he injected 1,500 c.c. of normal saline in thirty minutes. Gilligan and co-workers<sup>7</sup> observed venous pressure responses to intravenous normal saline or to a 5 per cent glucose salt solution in hospital patients without heart disease. They concluded that, although venous pressure variations are dependent on the volume of fluid injected, the increment of venous pressure increase did not necessarily follow the degree of blood dilution.

Studies indicate that the time interval, and the volume of fluid injected are the factors which may modify venous pressure responses. Caughey controlled the time interval without correcting for the volume of fluid injected. Gilligan

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studied blood dilution with variations in the time interval. No experiments are reported in which both the time and volume factors were controlled and studied. In this study either the time interval or the volume factor was constant, while the other variable was changed in order to determine the limits within which these factors influence venous pressure.

### METHOD

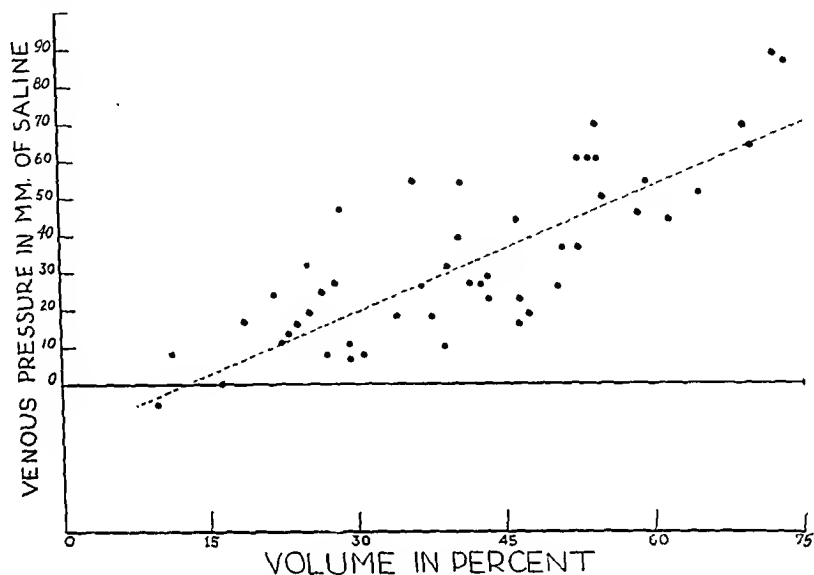
Normal healthy adult dogs were anesthetized with intraperitoneal injections of 30 mg. per kilogram of sodium pentobarbital.\* One and one-half inch No. 18 needles were used both for injection and measurement of venous pressure. Normal saline solution heated to body temperature was injected at a continuous rate into the vein of the foreleg by means of hydrostatic pressure from a graduated flask. Direct venous pressure measurements were made from the vein of the hindleg with a continuous drip saline manometer devised for the experiment. In a manometer of this type the respiratory movements may be observed continuously, while the constant flow of 15 to 20 drops of saline solution per minute prevented coagulation of the blood within the needle. At a rate of 15 drops per minute, 33 mm. of saline are necessary to overcome the resistance of the rubber tube and of the needle distal to the manometer. With the bottom of the manometer at the approximate center of the heart, the 33 mm. factor was subtracted from the observed readings in order to obtain actual venous pressure values. Respiratory fluctuations, and the velocity with which the added saline in the manometer dropped to the level at which pressures were in equilibrium, were the means used to check for obstruction within the measuring needle. The blood volume was calculated as one-eleventh of the body weight, and the normal saline injected is expressed as a percentage of this estimated volume. The change in venous pressure during the course of the experiment is expressed in millimeters of saline. In the shorter experiments venous pressure and volume inflow readings were observed every two or three minutes, whereas in the longer experiments these observations were usually checked every five minutes. A total of 83 experiments on 25 dogs were carried out. Ten control experiments in which venous pressures were measured for fifteen-minute intervals showed the normal variations under surgical anesthesia to be less than  $\pm 10$  mm. of saline. Fifty experiments were performed with the time interval fixed at fifteen minutes, while the inflow was changed. Another 23 experiments were carried out with the fluid inflow fixed at approximately 50 per cent of the calculated blood volume, while the time was varied.

### RESULTS

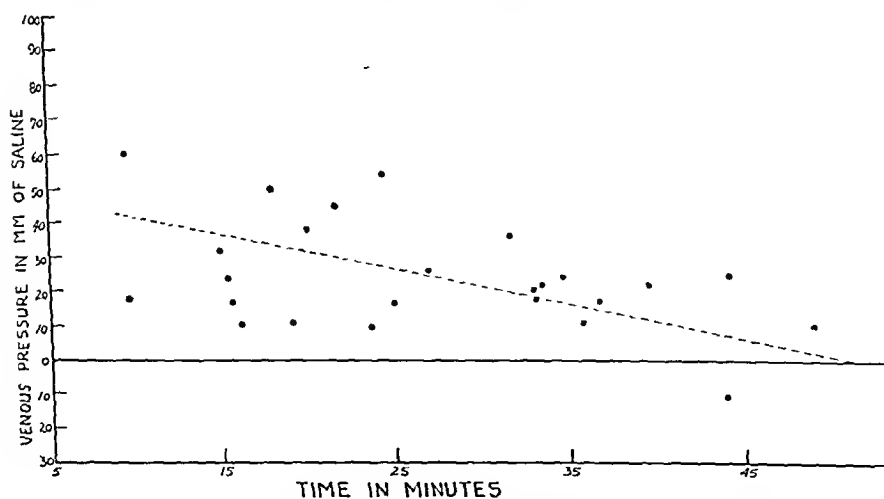
The results have been expressed in graphic form for sake of brevity, and to illustrate the variability of response in normal dogs under surgical anesthesia. Each point in Graphs 1 and 2 represents the total fluid inflow, and the change of venous pressure in millimeters of saline during the course of one experiment. The broken lines representing the most probable response have been drawn so as to divide the points equally.

\*Donated through the courtesy of the Abbott Laboratories.

Graph 1 illustrates that, with the time interval fixed at fifteen minutes, greater volumes of normal saline produce greater increments in venous pressure. Volumes greater than 10 per cent of the blood volume have to be injected in this time interval before any increase in venous pressure may be expected. Injections equal to 75 per cent of the blood volume in fifteen minutes produced the maximal response of venous pressure rise to 89 mm. of saline.



Graph 1.—Fluid injected expressed in percentage of estimated blood volume.

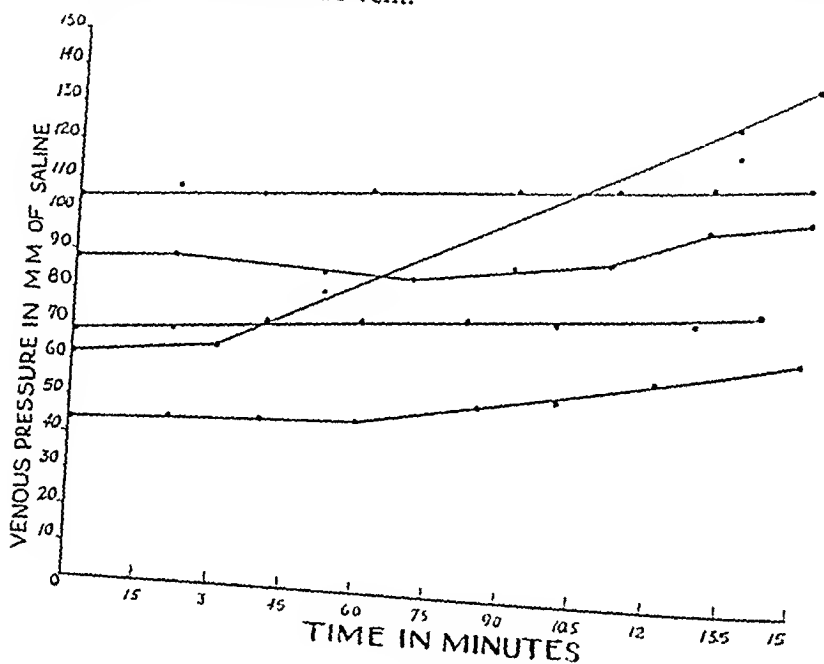


Graph 2.—Saline injected equals 50 per cent estimated blood volume. The volume is constant and the time interval is altered.

Graph 2 shows that with the amount of normal saline injected maintained at approximately 50 per cent of the blood volume; as the time interval of injection is prolonged, the increase in venous pressure becomes progressively less, so that beyond forty-five minutes an animal may show no increase in venous pressure.

Graph 3 shows five individual experiments (numbering from top to bottom) representative of the whole group.

The majority of the responses were either gradual increases or ones that showed abrupt breaks as do curves 4 and 5. Straight lines usually occur when the volume of fluid injected is less than 35 per cent of the calculated blood volume. Changes in the slope of the line develop when the volume injected is 40 per cent or more of the calculated blood volume. Increases in venous pressure appear to be directly proportioned to the volume of saline solution injected. Curves like 2 follow either alterations in velocity of fluid injection or an increase in resistance to the flow of saline from the manometer, due to the poor position of the needle within the vein.



Graph 3.—Individual responses.

#### DISCUSSION

The relationship between volume of fluid injected and the elevation in venous pressure appears more evident if the time interval is less than fifteen minutes. When the time interval is forty-five minutes or more, diffusion and vasodilatation can compensate fully, so that injections of normal saline equal to 50 per cent of the calculated blood volume may show no elevation in venous pressure. Injections of physiological saline at constant velocities will produce venous pressure increases closely approximating a straight line; shifting the velocity inflow in either direction will cause a corresponding change in the slope of the venous pressure curve. Even though different animals vary as much as 50 mm. of saline for the same volume of fluid injected, on repeated trials in any one dog the results agree closely. None of the animals developed cardiac decompensation following these injections or showed any deleterious effects on the following day.



Moderate pressure, produced by placing one's hand on the animal's chest, caused an increase of venous pressure from 4 to 15 mm. of saline. During the experiment the heart rate usually accelerated and the apex impulse became more forceful. If the animals shivered or stretched their extremities, venous pressures would increase from 10 to 30 mm. of saline. Respiratory movements showed that inspiration produced depression in the venous pressure measuring 2 to 5 mm. of saline, which returned to the original level during expiration. The majority of initial venous pressure readings of saline were between 50 and 70 mm., the lowest value was 29 mm., and the highest was 105 mm.

#### CONCLUSIONS

1. Both the volume of normal saline injected and the time interval are capable of altering the response of venous pressure to intravenous fluids.
2. With a fixed time interval of fifteen minutes, volumes less than 10 per cent of the calculated blood volume may cause no change in venous pressure.
3. Volumes of saline equal to 50 per cent of the calculated blood volume will show a slight or no increase in venous pressure if the time interval exceeds forty-five minutes.
4. Intravenous saline solution equal to 75 per cent of the calculated blood volume injected in fifteen minutes is tolerated without harmful effects.

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## CARDIOSPASM\*

### OBSERVATIONS ON THE USE OF PROSTIGMINE: A CLINICAL AND EXPERIMENTAL REPORT

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CLINICAL and experimental observations on the use of prostigmine as an aid in the treatment of cardiospasm and mega-esophagus are reported. The accepted therapy for cardiospasm in the past has been (a) gastric lavage; (b) dilatation of the cardia; (c) the use of antispasmodics. Psychotherapy, diathermy,<sup>1</sup> and vitamin B<sub>1</sub><sup>2</sup> have been reported to be of value. These measures have been effective to a degree in relieving the patient of discomfort and in improving his general nutrition. The esophagus has in most instances remained the same size, and patients have had recurrent symptoms but with a minimum of discomfort despite the large esophagus. Much evidence has accumulated recently to show that in a number of instances cardiospasm is related to damage to vagus fibers or to damage and loss of ganglion cells of the myenteric plexus of the esophagus at the cardia.<sup>3</sup>

The theory of Hurst and associates<sup>4</sup> proposed that following destruction of the parasympathetic myenteric plexus, the sympathetic nerves act unopposed and keep the cardia closed, so that the normal opening reflex of the cardia cannot take place at swallowing<sup>5, 6</sup> and dysphagia occurs, with gradual dilatation of the esophagus. This theory is supported indirectly by observations of Klee<sup>7</sup> on the cat: after section of the splanchnic nerves the cardia lost its power to open reflexly. This is comparable to the tonic "locking" of certain muscles in mollusks after sectioning of their motor nerve. The term "achalasia" is certainly better than "cardiospasm," although it may not cover the entire field of clinical experiences. It is difficult to conceive, *i. e.* that degenerative changes in the myenteric plexus of the esophagus occur in cases of functional obstruction of the cardia in hysteria; or in reflex disturbances from the abdominal viscera which sometimes improve after operation (cholecystitis, appendicitis, etc.). Recently, intractable cardiospasm has been relieved successfully by bilateral cervicothoracic sympathetic ganglionectomy.<sup>8</sup> This may be considered as proof of sympathetic achalasia, although a longer period of time is necessary for judging the permanency of the beneficial effects of the operation.

From the foregoing, drug therapy for achalasia and mega-esophagus can be elaborated on a logical basis, assuming that we are dealing with a true

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achalasia, that is, a condition in which degenerative changes have taken place in the myenteric plexus of the lower esophagus, through which the parasympathetic nerves effect normal peristalsis. Drugs that affect the parasympathetic system may be used to produce increased tonus and peristalsis of the esophagus, and thus push the food into the stomach. At first, we assumed that some parts of the plexus, upon which these drugs would act, were still functioning. Later we found that certain parasympatheticotropic drugs act not only on parasympathetic endings, but also on the smooth musculature directly (v.i.). Eserine and prostigmine were employed, the latter being the drug of choice because it lacks the disagreeable by-effects of the former.

We first made observations on two patients with very pronounced dilatation of the esophagus of eleven and twelve years' duration. The histories are briefly summarized below. At the beginning of the study we made certain that there was no organic stricture or stenosis of the esophagus. This is a precaution which we feel is necessary to emphasize.

M. F., female, aged 21 years, was admitted to the Michael Reese Hospital on May 8, 1938, because of right lower lobe pneumonia. During convalescence the patient reported that she had a "stricture" of the esophagus and difficulty in swallowing food. The history revealed that the symptom began rather suddenly at the age of 12 and was associated with a severe emotional conflict with her parents. At this time she was admitted to the Sarah Morris Hospital and treated for esophageal "stricture" by dilatation. After a short stay in the hospital her condition improved and she was discharged. In 1929 she was readmitted to Michael Reese Hospital because of a cough and loss of weight. A dilated esophagus was noted. Fluoroscopy and roentgen examination (July 21, 1929) showed mega-esophagus and cardiospasm. Following repeated dilatation of the cardia her condition again improved. Studies during the patient's convalescence on her last admission to the hospital on May 19, 1938, revealed a marked dilatation of the intrathoracic and apparently diaphragmatic portion of the esophagus, with a constriction just above the stomach. On May 27, 1938, an attempt to pass a dilator bougie through the cardio-esophageal hiatus was unsuccessful. The patient was given physostigmine per os 1/150 grain daily, with daily gastric lavage followed by dilatation with a Mosher dilator for one week. This made her feel better and she had less difficulty in getting the food to "go down." She was discharged from the hospital on June 16, 1938, and observation was continued in the clinic. At this time the following routine procedure was pursued: Dilatation was discontinued. Prostigmine\* was given by mouth, 15 mg. three times daily, and the patient was advised to return to the clinic in four weeks. At the end of this period she was reported to be improved. Swallowing of solids was better, and she did not need to drink fluids to force food down. Nevertheless, the patient requested dilatation since she felt that this procedure, in addition to the prostigmine, had helped her. Dilatations by the Mosher dilator, at approximately two-week intervals, and then at monthly intervals, were continued. Roentgenograms and fluoroscopic observations of the esophagus were made over the period, and it appeared that the esophagus was smaller.

We have observed this patient for over two years. During periods, when prostigmine was withheld, the patient would return with more complaints and ask that the drug be continued. This was true even though she had had periodic dilatation of the cardia. The patient had been dilated previously at other institutions, but she had not experienced the same degree of improvement as from prostigmine and dilatation. She had also undergone an extensive course

\*Supplied by Hoffmann-LaRoche Co.

of psychotherapeutic treatment at another institution with no apparent improvement. The patient admits that her symptoms are aggravated during severe emotional strain.

As a final check, we made fluoroscopic observations of the effect of the injection of prostigmine on the esophagus of this patient. Under fluoroscopic observation a barium meal was administered and 1 mg. of prostigmine was injected intramuscularly. Fifteen minutes later increased peristalsis and tonus (which reached its peak in twenty minutes) were observed. The increased contractions of the esophagus were particularly marked above the cardia, and the barium meal was emptied rapidly into the stomach. Intramuscular injections of 0.5 mg. of adrenaline hydrochloride had no visible effect on the esophagus.

R. N., female, aged 40 years, was admitted to the Michael Reese Hospital on August 6, 1937, after five days of repeated emesis. The past history revealed that she had a cholecystectomy in 1928, and subsequent to this had developed a large esophagus for which she had repeated dilatation with moderate relief. In 1933 the patient had a thyroidectomy. She was an emotional person, with quite a complicated social background. X-ray revealed an unusually large esophagus. The patient was placed on prostigmine, per os, 15 mg. three times daily, and later twice daily, and has been receiving same intermittently for approximately two years. At first she seemed to be distinctly improved, but as she became adjusted to the huge dilatation, it was most difficult to determine what symptomatic improvement we were obtaining. Both dilatation by Mosher dilator and prostigmine seemed to improve her condition, and like the other patient, she expressed the opinion that the prostigmine made her feel better. On the other hand, she had so many emotional conflicts that it was hard to evaluate the improvement.

The effect of prostigmine (and other drugs) on the esophagus was tested under fluoroscopic observation. At 9 A.M. 1/150 grain of atropine sulfate was injected intramuscularly. The patient's blood pressure was systolic 110 and diastolic 84 at 10 A.M. At 10:15 A.M. barium milk mixture was given, but the patient managed with difficulty to drink about  $\frac{3}{4}$  cup. The esophagus was seen fairly filled, with shallow peristaltic waves traveling downward. At 10:30 A.M. 1 c.c. of 1:2,000 ergotamine tartrate was administered intramuscularly. One minute later much deeper and much more frequent waves were observed moving down the esophagus than before. At 10:50 A.M. the blood pressure was systolic 100 and diastolic 70. More barium mixture was given, and shallow peristaltic waves were observed. One-half milligram of prostigmine was injected intramuscularly. The depth of the peristaltic waves increased within a few minutes, at times giving the impression of contraction rings traveling down the esophagus, and a greater frequency of peristalsis and increase of tone of the organ was observed. At 11 A.M. the blood pressure was systolic 118 and diastolic 85. One milligram of adrenalin hydrochloride was injected intramuscularly. Peristalsis, which after the injection of prostigmine had been present continuously, seemed to be somewhat increased. No untoward effect of the drugs given above was noted. At 11:10 A.M. the blood pressure was systolic 114 and diastolic 75.

We realize the difficulties in evaluating therapeutic results, particularly in this condition. It is well recognized that mere dilatation by any type of dilator will improve the well-being of a patient with achalasia, and will diminish the quantity of esophageal retention. It was our thought, that prostigmine, by acting on the parasympathetic plexus, would increase peristalsis and tone of the esophagus. Our observations definitely show that it does increase peristalsis, particularly in the lower portion of the esophagus. In that sense a propelling action of prostigmine is accomplished. Our patients stated that they were better with prostigmine and dilatation. It must be recognized that the achalasia and esophageal dilatation in our two patients were of long duration and of extreme

character. We suggest the use of eserine or prostigmine in early cases. Likewise, it is suggested that hypodermic medication rather than oral administration be tried in cases of chronic dilatation.

#### ANIMAL EXPERIMENTS

In order to check the effects of prostigmine on the esophagus, experiments were performed on dogs. The animals were anesthetized with nembutal, and three balloons were inserted into the esophagus at different levels: just above the cardia, into the midportion, and just below the glottis. The balloons were connected to water manometers and were inflated with about 5 cm. of water pressure. Blood pressure was recorded in the usual way with a mercury manometer connected to the carotid artery. In Experiment 1 (Fig. 1) respiration was not interfered with, and the curve showed its rhythmic effects on

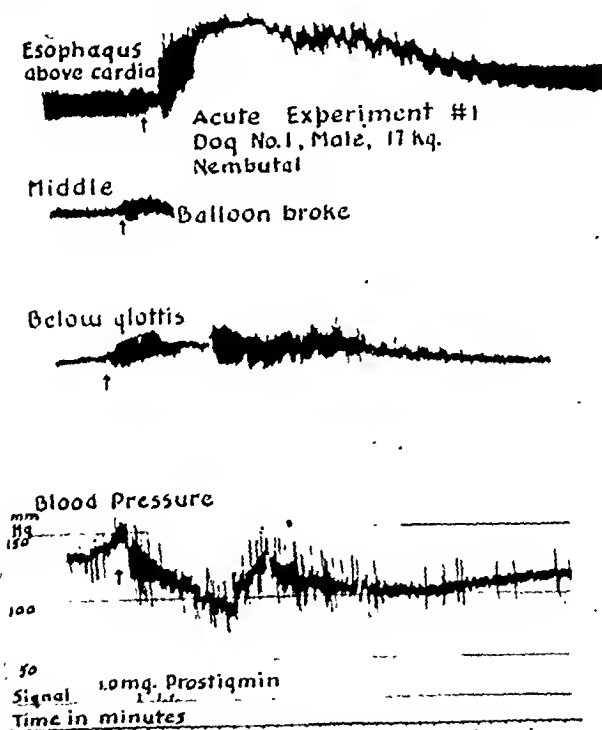


Fig. 1.

esophageal motility. One milligram of prostigmine injected intramuscularly produced a considerable increase of tone and peristalsis in the lower portion of the esophagus, lasting for about twenty minutes, while the upper portion of the esophagus was little affected. The animal in Experiment 2 (Fig. 2) was given artificial respiration, its chest was opened widely, and both phrenic nerves were cut in order to eliminate the effects of respiration on the esophagus. Injection of 1 mg. of prostigmine intravenously produced considerable rise of tone and peristaltic activity in the lowest portion of the organ, much less in the middle and very little in the upper portion. The effects lasted approximately fifteen minutes. In Experiment 3 (Fig. 3) the same operative procedure was followed

as in Experiment 2. Three-tenths milligram of prostigmine intravenously was followed by a rise of tone and motility of the lowest portion of the esophagus, lasting for about twenty-five minutes. The middle portion showed little, and the upper portion showed no effects of the drug. One hour later 1 gamma of acetylcholine hydrochloride was administered intravenously; it had little or no effect on the esophagus while a distinct transient drop of blood pressure occurred. Following this, 4 mg. of atropine sulfate were injected intramuscularly. Twenty minutes later 1 gamma of acetylcholine was injected again, and the negative response of blood pressure indicated that atropine had taken effect.

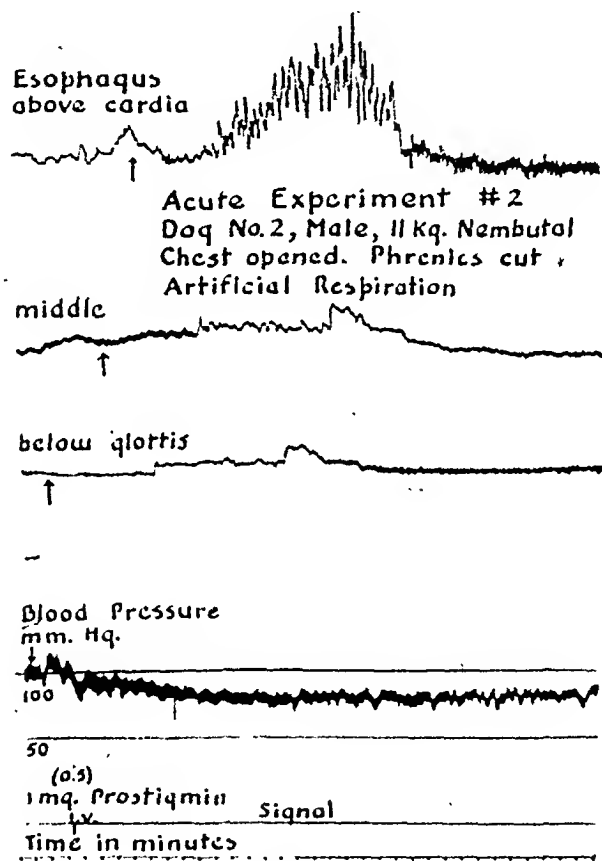


Fig. 2.

Following this, 0.3 mg. of prostigmine was injected intravenously; this dosage was repeated twenty minutes later. Each time a marked rise of tone and contractions of the lowest portion of the esophagus occurred, but the middle and upper portions did not respond. Fifteen minutes after the second injection of prostigmine, 1 gamma of acetylcholine was given intravenously, with no effect on blood pressure. This showed that the antagonistic effect of the atropine still prevailed.

These experiments demonstrated two facts to us: First, that prostigmine raises tonus and rate of peristalsis of the esophagus and acts mainly on its lower third. Second, that prostigmine acts on this organ not only through stimulation of its parasympathetic innervation, but also through a direct effect on its

musculature. We draw this latter conclusion from the fact that prostigmine still affected the organ after the parasympathetics were paralyzed by atropine, as proved by the ineffectiveness of acetylcholine administered repeatedly after atropine. It seems, therefore, that prostigmine is a useful drug in true achalasia in which more or less of the extrinsic and intrinsic parasympathetic innervation of the lower esophagus has been destroyed by pathologic processes. The observations on the dog agree with those obtained on the two patients reported in this paper. The effect of ergotamine on the second patient is of interest, because this drug is known to decrease and, in a sufficient dose, to abolish and reverse the typical effects of sympathetic stimulation. Since in achalasia there is a predominance of sympathetic innervation of the lower esophagus and cardia, it would seem logical to treat such patients with a combination of eserine or prostigmine and ergotamine. This is under experimental investigation at present.

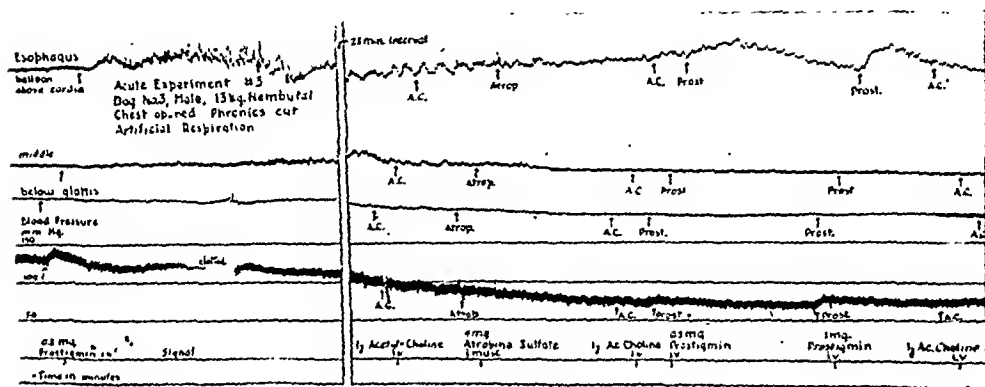


Fig. 3.

## SUMMARY

Two patients suffering from achalasia and dilatation of the esophagus were treated by oral administration of prostigmine. Both of them seemed to be benefited by this therapy. Fluoroscopic observations revealed that tonus and peristaltic rate of the esophagus were markedly increased following intramuscular injection of prostigmine. Injection of ergotamine into one patient likewise revealed increased tone and peristaltic rate of the esophagus. Since achalasia of the cardia has been assumed to be due to diminished or absent function of parasympathetic innervation and prevailing sympathetic innervation, prostigmine and ergotamine seem to offer a logical and promising method of treatment of this condition.

Experiments on anesthetized dogs confirmed the observations made on the patients and further reveal that prostigmine acts not only through stimulation of the parasympathetic innervation of the esophagus, but also through a direct effect on its musculature.

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## CHRONIC, ATROPHIC TYPE OF BRUCELLOSAL ARTHRITIS

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IT IS well established that different types of systemic infection may cause polyarthritides which, in its clinical appearance and symptomatology, is essentially indistinguishable from true atrophic arthritis. That is essentially true when such arthritis appears in women, in whom atrophic arthritis is most common.

Chronic brucellosis is in course of evolution. At least in the Southwest physicians are becoming increasingly "brucellosis conscious," and rightly so. In keeping with this general trend, and as chronic arthritis has a varied etiology, it is now part of good diagnostic routine to do agglutination and opsonocytophagocytic tests as well as a skin test of each patient for the presence of brucellosis. Reports as to the number of cases of chronic arthritis or chronic fibrositis having positive serologic and skin tests for brucellosis are the subject of a paper in process of preparation. Herein I set forth a syndrome occasionally found in which the pathology is from a clinical standpoint that of atrophic arthritis. Yet, the etiology is that of chronic brucellosis. The two cases presented are representative instances. They occurred in females. Both had typical fusiform spindle-shaped swellings of the interphalangeal joints of the fingers of both hands, as well as involvement of joint tissues in other parts of the body. Joint involvement, from the beginning, was essentially persistent and was not subject to regression. Its development was insidious and progressive. Presence of cold, clammy skin of both hands was noted. Past removal of apparent foci of infection had in general caused no regression of the arthritis. Physical therapy and other measures failed to relieve or to interrupt the disease course. A familial history of arthritis was absent. In one case knee and shoulder joints were definitely involved by a type of fibrous tissue swelling that is often seen in atrophic arthritis. An outstanding x-ray finding was that the bones of the involved joints did not reveal the narrowing of joint space and the decreased density of the ends of the bones forming the joints, which is seen



in cases of typical atrophic arthritis after the process has been present for some period of time, as was the case in these individuals. Laboratory tests revealed a lack of marked degree of secondary anemia that is often present in atrophic arthritis. Streptococcal antibodies were absent. Gastric analysis was normal. The gonococcal complement fixation test was negative. An active red blood cell sedimentation test was present. The Schilling differential count showed a mild increased shift to the left. An important clinical laboratory finding was a positive agglutination test for undulant fever.

The opsonocytophagic test also revealed a strongly positive test, being in one case as high as 100 per cent mild, moderate, and marked phagocytosis. In both cases skin test was strongly positive at the end of forty-eight hours and for a few days thereafter.

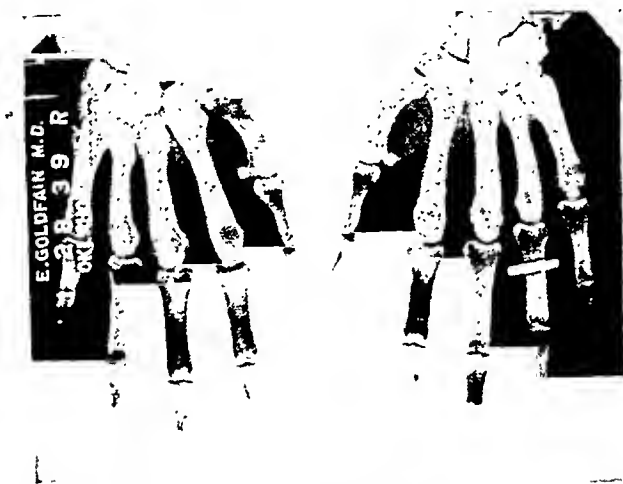


Fig. 1.—A, Case 1. M. R. B. X-ray of hands. Chronic arthritis, atrophic type, four years' duration. Note lack of joint space narrowing and demineralization. B, Same case as shown in Fig. 1. X-ray of right elbow. Note lack of bony pathology.

CASE 1.—Mrs. M. R. B. was first seen in the summer of 1935, when typical spindle-shaped fusiform swellings involving the interphalangeal joints of nearly all her fingers were present. Her physician husband was informed that it appeared to be a case of atrophic arthritis. She was carefully investigated in due time for focal infection, but none was found to be present. Streptococcal antibodies were not present. Agglutination tests for undulant fever were strongly positive. The opsonic index test was positive. The skin test was strongly positive.

X-ray examination of the involved joints of the fingers simply revealed the normal bones that form the involved joints. Diffuse soft tissue swelling could be seen by x-ray shadow. X-ray examination made of her hands in January, 1939, four years after the onset of her trouble, revealed normal joint spaces and bones forming the involved joints, except for very slight lipping in the terminal joints of both fifth fingers. Narrowing of joint space and demineralization, such as one sees in atrophic arthritis, particularly atrophic arthritis present over such a long period of time, were absent.

X-ray examination of the right elbow joint was made in September, 1937, because of joint symptoms, but it failed to reveal any bony pathology.

The patient's past history was essentially negative, except for influenza and a miscarriage in 1927. An appendectomy and curettage were done in 1927, as was the removal of one cystic ovary. In the latter part of 1927 chills at intervals appeared, accompanied by wringing sweat and a feeling of stiffness all over her body, the stiffness involving most

markedly both upper extremities, so that for a period of time they could be used only very little; other joints of her body were involved. A tonsillectomy was performed in 1927. Very slowly and gradually she became better of her rheumatism, but a marked nervous condition developed. Treatment for her nervous condition resulted in gradual clearance of her rheumatism. Prior to the onset of the typical symptoms of arthritis in her hands, she felt ill for a long period of time and lost weight. The patient is of the age during which hypertrophic arthritis develops.

As a result of brucella bacterin therapy administered by her physician husband over a period of a year she improved in every way, clinically and from the standpoint of x-ray examination and laboratory findings.

The absence of findings, laboratory, x-ray, or clinical, that would explain her atrophic arthritis, plus positive skin, agglutination, and opsonic index tests for brucellosis, as well as her admirable response to treatment for chronic brucellosis, with no definite relapse after treatment had been completed, justifies labeling this case as one of brucello-al arthritis, chronic, atrophic type. The case illustrates the chronicity of illness that may be present in individuals plus the essential relapses and improvements that occur. This may happen in atrophic arthritis.



Fig. 2.—Case 2. Mrs. R. S. X-ray of hands. Chronic arthritis, atrophic type. Note lack of demineralization and articular cartilaginous involvement.

CASE 2.—Mrs. R. S. was first seen in January 14, 1937. Her history dated back two years, beginning with pain and discomfort in the left shoulder joint. These symptoms appeared in spells at irregular intervals over a period of some few months. Moderate response at first to salicylates occurred. Thereafter the arthritis appeared insidiously, and became worse progressively. Many joints became involved, especially the hands, knees, and feet, as well as the shoulders and elbows. She had some teeth extracted and her tonsils removed. She had taken treatment at bathing resorts. Scarlet fever and whooping cough occurred in childhood. She has had two pregnancies, one of which was aborted with no ill effects. The abortion took place after the onset of her arthritis. The family history is negative.

Physical examination was negative, save for the presence of marked motion limitation of both shoulder joints and right elbow. Cold, clammy perspiration was present in both hands. Wrist and interphalangeal finger joints were indurated. The involved finger joints revealed typical spindle-shaped fusiform swellings seen in atrophic arthritis. Knuckle joints were also enlarged. A low-grade inflammatory infiltration of the fibrous tissues of both knees, with moderate restriction of flexion, was present. Lateral pressure over the phalangeal joints caused pain. Adherent tubes were questionably present on pelvic examination.

X-ray examination revealed a lack of joint space narrowing and an absence of demineralization of the bones forming the joints. A faint shadow of the soft tissue swelling was noted.

Laboratory findings revealed absent streptococcal antibodies and negative gonococcal complement fixation test. Gastric analysis, blood count, urinalysis, and stool examination were negative. The red blood cell sedimentation test showed a moderately active curve. Agglutination test for undulant fever showed strongly positive results in dilutions of 1:50. Opsonic index test for undulant fever revealed mild, moderate, and marked degree of opsonocytophagocytosis to 100 per cent. The skin test for undulant fever was strongly positive 4+ at the end of forty-eight and ninety-six hours.

The patient responded well to brucellosis treatment and at the end of two years is practically cured.

Diagnosis: Brucellosal arthritis, chronic, atrophic type.



Fig. 3.

#### SUMMARY

1. The two patients presented had clinical objective and subjective findings that would stamp them as cases of atrophic arthritis.

2. X-ray findings, even though the condition had been present in each case for a long period of time when first seen, and for at least two years thereafter in one case and four years thereafter in the second, continued to reveal none of the findings characteristically seen in true atrophic arthritis.

3. From a laboratory standpoint findings that are usually noted in atrophic arthritis were absent, save for the presence of a quite active sedimentation test in each instance. Both cases revealed strongly positive agglutination, opsonocytophagic, and skin tests for brucellosis.

4. Each patient has responded quite satisfactorily to management for chronic brucellosis: one case, Mrs. R. S., as soon as she improved on treatment for chronic brucellosis, discontinued treatment. The condition, however, reappeared, and continuation of proper treatment has resulted in practical cure.

#### CONCLUSIONS

1. Two cases are here presented which are typically atrophic arthritis from a clinical standpoint.

2. Laboratory findings are presented which indicate that the arthritis was due to chronic brucellosis.

3. X-ray findings are set forth indicating that chronic brucellosis does not cause articular cartilage destruction nor epiphyseal bone involvement such as is in due time present in practically all cases of atrophic arthritis.

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## MULTIPLE INVASION OF THE BLOOD STREAM\*

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IT IS well known that bacteriologic study of infected tissue may disclose more than one organism. Kolle and Hebsch,<sup>1</sup> and Ford,<sup>2</sup> described many such cases and developed an elaborate classification. However, there has been no systematic study of multiple invasion of the blood stream. A few references have been made to cases of septicemia complicated by a secondary bacteriemia, but we have seen no report devoted to the subject of multiple blood stream infection.

In 1906 Libman<sup>3</sup> reported 98 cases of sepsis, among which were several instances of multiple infection. The combinations were: streptococcus + gonococcus, streptococcus + *B. proteus*, *B. coli* + streptococcus + *Micrococcus albus*, *Staphylococcus aureus* + *pyocyaneus*, and three cases with staphylococcus + streptococcus. Scott<sup>4</sup> reported a series of 80 patients with urologic disorders who had had routine blood cultures taken with each chill. They found five (6 per cent) who had more than one organism in the blood at the same time. The combinations were staphylococcus or streptococcus with either *B. proteus* or *B. pyocyaneus*. Neuhoﬀ, Amfses, and Hirshfeld<sup>5</sup> reported a series of 150 patients with sepsis. In only one patient was there a multiple infection, a patient with both streptococci and staphylococci in the blood. Adams<sup>6</sup> reported a series of 12 patients with sepsis, two of whom had multiple blood invasion; in one patient an embalming wound resulted in a fatal septicemia with *Streptococcus hemolyticus* and *Vibrio septique*; and in another patient, incision of an area of cellulitis was followed by a fatal sepsis with staphylococcus and *Streptococcus hemolyticus*. Similar series were reported by Shanks and Khan,<sup>7</sup> Warren and Herrick,<sup>8</sup> Cadham,<sup>9</sup> Trask,<sup>10</sup> and by Doane and Cates.<sup>11</sup>

The following is a summary of the combinations described by the aforementioned authors:

|  |   |    |
|--|---|----|
| Streptococcus + gonococcus                                   | ~ | 1  |
| Streptococcus + <i>B. proteus</i>                            | ~ | 1  |
| Streptococcus + <i>B. coli</i> +<br><i>Micrococcus albus</i> | ~ | 1  |
| Staphylococcus + <i>B. pyocyaneus</i>                        | ~ | 2  |
| Streptococcus + staphylococcus                               | ~ | 11 |
| Streptococcus + <i>Vibrio septique</i>                       | ~ | 1  |
| <i>B. typhosus</i> + enterococci                             | ~ | 5  |
| Streptococcus + <i>B. typhosus</i>                           | ~ | 1  |
| Streptococci + <i>B. coli</i>                                | ~ | 1  |
| <i>B. proteus</i> + <i>B. coli</i>                           | ~ | 1  |
| Staphylococcus + <i>B. coli</i>                              | ~ | 1  |
| Staphylococcus + <i>B. influenzae</i>                        | ~ | 1  |
| Pneumococcus + influenzae                                    | ~ | 2  |
| Staphylococcus + pneumococcus                                | ~ | 2  |
| Staphylococcus + pneumococcus + <i>B. coli</i>               | ~ | 1  |

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On the other hand, many authors present large series of septic patients where there is no mention made of multiple infections. These include Bean,<sup>12</sup> who reports 109 patients; Crocker, Valentine, and Brody,<sup>13</sup> who report 52 patients; Dennett<sup>14</sup> and Thelander,<sup>15</sup> who report, respectively, 115 and 32 cases in children; Glass,<sup>16</sup> 63 cases; and Kohner,<sup>17</sup> 282 cases. This makes a total of 652 patients with sepsis in whom no instance of multiple infection is mentioned.

In this paper we are presenting 24 children who showed more than one organism in the blood stream during their period of hospitalization. These cases were collected over a five-year period and represent 8 per cent of all patients with bacteremia studied during this time.

We found it best to classify these patients as follows:

- (1) True mixed septicemia.
- (2) Septicemia complicated by a secondary bacteremia.
- (3) Spurious mixed septicemia.
  - a. Where the second organism is a variant of the first.
  - b. Where a casual invader appears in the course of a sepsis.

#### METHOD

All patients with fever admitted to the pediatric wards between the years 1931 and 1935 had blood culture studies. The blood cultures were taken according to standard methods by an intern assigned to the service. All work was supervised by one individual, giving the results a good degree of uniformity throughout the years during which these cultures were taken. Fourteen cubic centimeters of the patient's blood were drawn and poured into culture media at the bedside. Five cubic centimeters were injected into each of two 200 c.c. Erlenmeyer flasks. The first flask contained 125 c.c. of veal infusion broth at pH 7.2; the second flask contained 125 c.c. of 2 per cent dextrose veal infusion broth of the same hydrogen-ion concentration. The remaining 4 c.c. were divided equally to form two pour plates, one with a 2 per cent dextrose veal infusion agar, and the other with a tube of plain veal infusion agar. Daily transplants were made from the two flasks, and smears were stained at the same time.

The invasion was considered "large" if there were more than ten colonies per cubic centimeter of blood. It was called "small" if only the liquid media showed growth. The size of invasion was "medium" if the broths were positive, but there were less than ten colonies on the plates.

#### I. TRUE MIXED SEPTICEMIA

By the term "true mixed septicemia" we refer to a type of sepsis where more than one organism is found in the same blood culture. There were nine patients in this group. The combinations of organisms were as follows:

|   |         |
|---|---------|
| 1. <i>Streptococcus hemolyticus</i> + <i>B. coli</i>                                | 4 cases |
| 2. <i>Streptococcus hemolyticus</i> + <i>B. coli</i> + <i>Staphylococcus aureus</i> | 1 case  |
| 3. <i>Streptococcus hemolyticus</i> + <i>Staphylococcus aureus</i>                  | 2 cases |
| 4. <i>Streptococcus hemolyticus</i> + <i>Pneumococcus</i> type XXI                  | 1 case  |
| 5. <i>Staphylococcus aureus</i> + <i>B. coli</i>                                    | 1 case  |

The number of colonies isolated of each organism was recorded as "numerous" in five instances; "large" and "medium" in two; "small" in one, and "unknown" in one.

In six patients of this group the blood cultures were performed post mortem. These patients lived for such a short time, or were so desperately sick that a culture was not taken during life. In each instance a cardiac puncture was performed immediately after suspension of respiration, and the blood was poured into media at the bedside.

The significance of the blood cultures in these cases depends on what significance can be placed on post-mortem cultures. If the blood after death is rapidly invaded with many organisms which have no relation to the disease process, then these cases are of no practical significance. In our experience, however, such rapid nonspecific growth does not ordinarily occur. Of 1,309 patients from whom we cultured specimens over a period of five years, 171 had the blood drawn immediately after death. Of these, fully 50 per cent were sterile. We agree with White<sup>18</sup> in supposing that these post-mortem cultures reveal the etiologic agents of disease which cannot grow out during life, but multiply rapidly after death because the immunologic resistance of the body is no longer intact.

Most patients in this group were very young. Three patients were less than a month old, four were less than 6 months old, one was 3 years, and one was 6 years old. There were four girls and five boys. Short case histories of these nine patients are given, but certain facts in the clinical course are particularly striking. Six patients were admitted to the hospital in a markedly toxic state and died within twenty-four hours. The other three died within four days after admission. The majority suffered from debilitating illnesses before admission. Six had been sick for one month or more, and three had been ill for a period of two weeks. In two patients the septicemia followed an operation. One patient had had a herniotomy; the other had an abscessed kidney removed. The most constant symptom was diarrhea, which occurred in six of the nine patients. The case histories follow:

CASE 1.—H. K., a 2-month-old male, was operated upon for an inguinal hernia because of mild obstructive symptoms for four days. He left the hospital on the tenth day following operation but returned two days later with marked diarrhea and dehydration. He died soon after readmission. Cardiac puncture immediately after death revealed 34 colonies per cubic centimeter of blood of *Streptococcus hemolyticus*, and 35 colonies of *B. coli*. Autopsy revealed partial atelectasis and empyema, lipoid pneumonia, parenchymatous degeneration of the liver, kidneys, and adrenals, and a well-healed operative scar.

CASE 2.—M. S., a 3½-year-old girl, was admitted with acute lymphatic leucemia. One week after admission her blood culture was sterile, but ten days later she developed acute parotitis and died. Cardiac puncture immediately after death revealed innumerable colonies of both *Streptococcus hemolyticus* and *B. coli*. Autopsy report confirmed the clinical diagnosis.

CASE 3.—G. T., a 6-month-old girl, was admitted with acute rhinopharyngitis, diarrhea, marked dehydration, and rickets. She seemed to improve for a few days with treatment, but at the end of the second week she developed cellulitis of the back and lapsed into acidosis and dehydration again. This time she did not rally and nine days later she died. Terminally, she developed the signs of a diffuse bronchopneumonia. Cardiac puncture immediately after death showed numerous colonies of *Streptococcus hemolyticus* and pneumococcus type XXI.

CASE 4.—I. B., a 3-week-old male infant, was admitted after having been ill for two days with acute rhinopharyngitis, marked cervical adenitis, and cellulitis of the neck. Four days later there was fluctuation in the area of cellulitis, and it was incised and drained. The child developed a marked diarrhea which persisted for three weeks. He then developed a diffuse bronchopneumonia and died. Blood culture on admission was sterile, but a cardiac puncture immediately post mortem showed numerous colonies of *Staphylococcus aureus* and *Streptococcus hemolyticus*.

CASE 5.—B. S., a premature female infant, was admitted from the nursery on the fifteenth day after birth, with an acute upper respiratory infection and diarrhea. Despite treatment the child grew steadily worse, developed signs of thrombosis in the cranial sinuses, and died. On admission the blood culture was sterile but cardiac culture immediately after death showed *Streptococcus hemolyticus* and *B. coli*. Autopsy revealed fetal atelectasis, passive congestion of the viscera, gastro-enteritis, focal pneumonia, focal necrosis of spleen and suprarenals, and thrombi in the cranial sinuses.

CASE 6.—L. R., a 3-month-old male, was admitted with diarrhea and marked dehydration, after having been sick with an acute upper respiratory infection for two weeks. With treatment the dehydration seemed to improve, but the patient developed a cellulitis of the left hand, and a day later, gangrene of the right hand. Blood culture showed *Streptococcus hemolyticus* and *B. coli*. The patient died three days later. Autopsy showed cellulitis of the left hand, gangrene of the right hand, marked cerebral congestion, cloudy swelling of the kidneys, liver, and heart, and multiple abscesses of the lung and kidneys. Culture of these abscesses showed the presence of *Staphylococcus aureus* and *Streptococcus hemolyticus*.

CASE 7.—L. B., a 6-year-old girl, was admitted with a history of chills and fever for one week. On admission she was markedly delirious. There were numerous purpuric areas on her body. She died the next day. Culture on admission, twenty-four hours before death, showed many colonies of *Streptococcus hemolyticus* and a *Staphylococcus aureus* in the blood.

CASE 8.—E. F., a 4-month-old male infant, was admitted with a history of a hematuria and a loss of weight for a month. After study an operation was performed. The child's course was progressively downhill, and he died a month after admission. The child showed *Staphylococcus aureus* in the blood twice, and a small invasion with *Staphylococcus aureus* and *B. coli* once, following the first cystoscopy.

CASE 9.—I. M., a 13-day-old male child, who was perfectly normal for six days after birth, developed a severe diarrhea on the seventh day. On the eleventh day he was admitted to the pediatric service with dehydration and gastrointestinal intoxication. At this time, he also had a bilateral purulent acute otitis media and a definite rhinopharyngitis. A continuous intravenous solution of glucose and a blood transfusion were administered, but the patient grew steadily worse, and two days later died. A cardiac puncture was done immediately after death, and a culture of the blood showed innumerable colonies of *Streptococcus hemolyticus*, *B. coli*, and a *Staphylococcus aureus*. Autopsy revealed an acute enteritis, purulent otitis media, lobular pneumonia, and cloudy swelling of the viscera.

## II. SEPTICEMIA COMPLICATED BY SECONDARY BACTERIEMIA

There were five patients in this group. In three instances the patient first had a *Staphylococcus aureus* sepsis and then developed a hemolytic streptococemia. In one case of *B. coli* septicemia, a secondary invasion by *Streptococcus hemolyticus* occurred. The fifth was a patient who was admitted with hemolytic streptococcus sepsis and later developed *B. coli* septicemia.

In three cases the size of the original invasion was classed as "large," and in two it was designated as "medium." The number of colonies in the secondary invasion was "large" in four instances, and "small" in one.



Four of the five patients in this group were less than 6 weeks old. One was 18 months old. All five were boys. All died.

The incubation period varied from three to fourteen days. The shortest period of hospitalization was twenty-four days, and the longest period of hospitalization was fifty-five days. In three cases there was an afebrile interval between two septic periods. In these it appeared as though the patients were convalescing from the first blood stream invasion when the second invasion began. The duration of the first acute episode was from eleven to sixteen days, and of the second, from seven to eight days.

In three patients an extensive erysipelas or an erysipelas-like eruption heralded the appearance of the secondary streptococcemia. In one, the entire neck was involved. In another, the lesion spread over the entire chest. In the third, there was involvement of both shoulders and the scrotum. We hesitate to designate these diffuse, bright red, indurated skin infections as erysipelas, because in all instances there were large numbers of colonies of *Streptococcus hemolyticus* in the blood. In all of our cases of primary erysipelas, the blood culture was usually sterile or at most an occasional small, transitory invasion was found.

We could not find any valid factor to account for the onset of the secondary infection. Treatment of the original sepsis may have created new foci of infection. In all instances the primary sepsis was treated vigorously with blood transfusions, clyses, infusions, and in one case with staphylococcus toxoid. In addition, suppurative foci were incised. On the other hand, we have 315 cases of sepsis that received similar treatment without developing secondary bacteremia. The case histories of the five patients in group II follow:

CASE 10.—A. S., a male, 3 weeks old, was admitted with a history of osteomyelitis of the right hip for ten days and of the right knee for two days. On admission he showed a diffuse pustular eruption and evidence of trauma to the scalp from a forceps delivery. Blood culture showed ten colonies of *Staphylococcus aureus* per cubic centimeter of blood. Some skin abscesses formed which required incision and drainage.

Supportive treatment was administered. The temperature subsided gradually and remained normal for a period of two weeks. The number of colonies in the blood stream gradually decreased, and after the third culture the blood was sterile. Toward the end of the third week after admission, there was a sudden elevation to 102° F.; swelling and redness of the left shoulder appeared at the same time. The shoulder was incised on the following day. Instead of a pure culture of *Staphylococcus aureus*, which had previously been found in all cultures of the skin lesions, there was now a mixture of staphylococcus and *Streptococcus hemolyticus*. The temperature, however, remained normal for four days; on the fifth day there was a rise, and the blood culture showed four colonies of *Streptococcus hemolyticus* per cubic centimeter of blood. The temperature continued to increase. Two days later there were 21 colonies of *Streptococcus hemolyticus* per cubic centimeter of blood, and on the tenth day after incision of the shoulder an erysipelas-like rash appeared over the shoulders and scrotum. Two days later the child died.

CASE 11.—B. H., a newborn baby boy, was well until the third day after birth, when he developed slight jaundice, fever, and a moderate diarrhea. On admission to the pediatric service on the sixth day of life, there were 35 colonies of *B. coli* per cubic centimeter; nine days later there was *B. coli* in one broth only. Moderate variations in the extent of invasion continued. One month after admission the culture was sterile and remained so for over a week. During this time the patient's clinical status seemed fair. At the end

of the fifth week there was a sudden rise in temperature to 104° F., and on the same day an erysipelas-like eruption appeared over the child's chest, and *Streptococcus hemolyticus* appeared in two broths. The rash spread, but the blood culture on the next day was still sterile. The child died four days later. Autopsy confirmed the diagnosis of erysipelas.

CASE 12.—N. E., an 18-month-old male baby, was admitted with a history of coryza and generalized anasarca of two weeks' duration. On admission, besides the edema, there was marked abdominal distention. Blood culture showed seven colonies of *Streptococcus hemolyticus* per cubic centimeter. Two days later the blood culture was sterile. On the sixth day an erysipeloid rash appeared over the lower back. The child had a septic temperature during the first two weeks, followed by an afebrile period of twenty days. At the end of that time he suddenly developed fever and diarrhea, and within twenty-four hours, intestinal intoxication. He died three days later. Cardiac puncture immediately after death showed innumerable colonies of *B. coli*.

CASE 13.—F. C., a male, 5 days old, was born at home after a difficult delivery. The infant was cyanotic and dyspneic for two days after birth. On the day of admission he became stuporous and gradually lapsed into coma. On examination a left Erb's palsy and evidence of intracranial injury was found. In addition there was a diffuse induration of the skin of the back. On the eleventh day after admission, the child's temperature became elevated, the edema of the back became red, and a mass appeared on the right buttock. Blood culture showed 20 colonies of *Staphylococcus aureus* per cubic centimeter. Two days later both abscesses were drained. The child's condition, however, did not improve, and six days later he died. Two days before death 50 colonies of hemolytic streptococci per cubic centimeter appeared in the blood.

CASE 14.—V. de P., an 8-day-old male, was admitted with jaundice and fever of a few hours' duration. On admission he was markedly jaundiced and somewhat cyanotic. A mild omphalitis was found. Blood culture showed *Staphylococcus aureus* in both broths. The child received supportive measures, but seven days later he still had 12 colonies of *Staphylococcus hemolyticus* in the blood. During the third week of his stay he developed evidence of a bronchopneumonia, but the blood culture was sterile. After this the course was steadily downhill. He developed diarrhea, and one week later cellulitis appeared at one of the transfusion sites, but this seemed to subside with hot compresses. Five days later an erysipeloid rash appeared on the scrotum and upper thighs. Blood culture showed 25 colonies of hemolytic streptococcus per cubic centimeter. The next day the infant died. Autopsy showed bronchopneumonia, cellulitis of scrotum and thigh, and acute lymphadenitis of nodes around the left common iliac vessels.

### III. SPURIOUS MIXED SEPTICEMIA

In this group of patients the clinical features were determined by the primary rather than by the secondary invader.

The secondary invader was one of two types:

- (A) *Streptococcus gamma* or *viridans* present in the blood as a casual invader, with no clinical or bacteriologic significance or
- (B) The second organism was a variant of the first.

The bacteriologic details of group A are shown in Table I.

The details of the cases in group B are indicated in Table II.

Cases 20, 21, and 23 might also be in group A, but we felt that it was more suitable to place them in this group. In Cases 22 and 23 the so-called "degraded" form of the organism appeared in the blood cultures before the "virulent" form.

The clinical significance of these two types of secondary invaders is not known. In the first group (group A) we can see no more significance than we

see in the occasional culture of a gamma streptococcus in the blood of patients with an acute infectious disease. The mutation from the virulent to the non-virulent form of an organism makes the prognosis more favorable (Cases 19, 20, 21), whereas the reverse usually spells a fatal outcome (Cases 22, 23).

TABLE I

| CASE NO. | PRIMARY ORGANISM                        | SIZE OF INVASION | CASUAL INVADER                | NO COLONIES | OUTCOME   |
|----------|---|------------------|-------------------------------|-------------|-----------|
| 15       | <i>Staphylococcus albus hemolyticus</i> | Medium           | <i>Streptococcus gamma</i>    | Small       | Recovered |
| 16       | <i>B. coli</i>                          | Large            | <i>Streptococcus viridans</i> | Small       | Died      |
| 17       | <i>B. coli</i>                          | Unknown          | <i>Streptococcus gamma</i>    | Small       | Died      |
| 18       | <i>Staphylococcus</i>                   | Large            | <i>Streptococcus gamma</i>    |             | Died      |

TABLE II

| CASE NO. | PRIMARY ORGANISM                         | SIZE OF INVASION | SECONDARY INVASION                          | SIZE OF INVASION | OUTCOME   |
|----------|--|------------------|---|------------------|-----------|
| 19       | <i>Staphylococcus aureus hemolyticus</i> | Medium           | <i>Staphylococcus aureus nonhemolyticus</i> | Medium           | Recovered |
| 20       | <i>Streptococcus hemolyticus</i>         | Small            | <i>Streptococcus viridans</i>               | Small            | Recovered |
| 21       | <i>Streptococcus hemolyticus</i>         | Medium           | <i>Streptococcus gamma</i>                  | Small            | Recovered |
| 22       | <i>Streptococcus viridans</i>            | Small            | <i>Streptococcus hemolyticus</i>            | Large            | Died      |
| 23       | <i>Streptococcus viridans</i>            | Small            | <i>Pneumococcus XX</i>                      | Medium           | Recovered |
| 24       | <i>Dysentery-like organism</i>           | Unknown          | <i>B. coli</i>                              | Unknown          | Died      |

## CONCLUSION AND SUMMARY

In 8 per cent of a total of 320 cases of septicemia, a second organism appeared in the blood stream at some time during the illness. In 3 per cent, two organisms of definite pathogenicity appeared in the same culture. In 1.5 per cent invasion by the second organism appeared as a complication of the original sepsis, often when the first infection seemed to be on the decline. In the remaining cases (3.5 per cent) the second invader seemed to have no clinical or pathologic significance, but appeared merely as a casual invader of the blood stream or as a variant of the first organism.

One might suppose that once the body resistance to blood stream invasion is overcome, any number of organisms could follow the first invader. If we may look upon sepsis as an infectious disease, we see that septicemia is not complicated by a superimposed blood stream invasion any more often than other infectious diseases. It would appear that the immunologic changes which permit the onset of a sepsis are specific for one organism. Invasion by more than one organism

rarely takes place. When it does occur, it is seen only in very young infants and the outcome is invariably fatal.

Two points of special clinical interest are worthy of note. The first is the appearance of an erysipeloid rash when a hemolytic streptococemia is superimposed on an existing sepsis. The other is the difference in the clinical course in types I and II. In the cases with two organisms in the same blood culture the incubation period was long, followed by a short, acute course terminating rapidly in death. Where the second invasion complicated a primary sepsis, the incubation period was short. The illness was characterized by two septic periods separated by an afebrile period, the second coincidental with the second invasion.

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## OBSERVATIONS ON THE TOXICITIES AND CHOLERETIC ACTIVITIES OF CERTAIN BILE SALTS\*

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IT IS a well-known fact that the toxicity of bile is due to its bile salt content.<sup>1-6</sup> The choleretic properties of bile salts are also known.<sup>7-13</sup> Comparisons of the relative toxicities and choleretic activities of the various bile salts have been limited by the fact that many of them have not been available in pure form. Some of the contradictory results which have been reported may be attributed to the fact that preparations of different degrees of purity have been used. We have recently had available a number of highly purified bile acid preparations and have studied their toxicity and choleretic activity in an attempt to correlate these properties with chemical structure.

### METHODS

The toxicities of seven bile salts have been compared by blood pressure studies on anesthetized dogs and by in vitro hemolysis studies on human erythrocytes. The relative choleretic activities have also been studied in the anesthetized dog.

*Bile Salt Preparations.*—The sodium salts of cholic acid, dehydrodeoxycholic acid, apocholic acid, and deoxycholic acid were prepared by dissolving the pure bile acids† in alcohol, neutralizing with dilute sodium hydroxide to the phenolphthalein end point, evaporating to dryness, and finally dissolving the dry salt in distilled water. Dehydrocholic acid‡ was furnished us as the sodium salt. We have used two different preparations of sodium glycocholate and of sodium taurocholate, one a natural product‡ and one a synthetic product.§ Aqueous solutions of the natural products were yellow in color whereas the synthetic salts gave clear colorless solutions.

Chemical assays revealed a 56 per cent cholic acid content for the natural taurocholate, corresponding to 71 per cent purity; the synthetic taurocholate assayed 80 per cent cholic acid, corresponding to 100 per cent purity; the synthetic glycocholate assayed 87 per cent cholic acid, corresponding to 97 per cent purity.

*Blood Pressure Studies.*—Dogs under sodium pentobarbital anesthesia were used. A tracheal cannula was inserted, and the carotid blood pressure was recorded. The common bile duct was cannulated close to the duodenum, and the

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cystic duct was ligated. The sodium salts of the bile acids in 10 per cent solution were used. All injections were given into the femoral vein at a standard rate (fifteen seconds). The doses arbitrarily chosen were 30 mg. per kilogram, which is the standard intravenous dose of sodium dehydrocholate in man, 10 mg. per kilogram, and 5 mg. per kilogram. Several injections were given to the same animal. At least twenty to thirty minutes were allowed between each injection, which was ample time for the blood pressure to return to normal. The order in which the different salts were given was varied in each animal.

*Hemolysis Studies.*—Hemolytic activity was studied by the method of Ponder<sup>44</sup> using human cells. Solutions containing 50 mg. of bile salt in 4 c.c. of 0.9 per cent sodium chloride solution were made up. Dilutions of this solution were then prepared: 1:10, 1:100, 1:1,000, etc. One cubic centimeter of standard suspension of human red blood cells was added to 4 c.c. of bile salt solution, and the time for complete hemolysis was noted. The tests were made in a water bath at 38° C.

*Choleretic Studies.*—The dogs were prepared as described for the blood pressure studies. After a uniform bile flow was established, a control period of thirty minutes to one hour was recorded. Thirty milligrams per kilogram of bile salt in 10 per cent solution was then injected into the femoral vein, and the bile volume was recorded every fifteen minutes until the rate of secretion had returned to the control level.

## RESULTS

*Blood Pressure Studies.*—The averaged results of the blood pressure studies are given in Table I. A 10 mg. per kilogram dose of each of six salts was tested on 10 animals. The 30 mg. per kilogram dose was tested on 10 animals with four salts and on 5 animals with two. Because of the limited supply of sodium taurocholate available, only a single dose, 30 mg. per kilogram, was tested on 6 animals. A 30 mg. per kilogram dose of synthetic sodium glycocholate was tested in 6 experiments on 3 animals. In 4 animals a 5 mg. per kilogram dose of apocholeate and deoxycholeate was tested.

As indicated in Table I, sodium dehydrocholate was the least toxic and sodium deoxycholeate was the most toxic of the seven salts tested. There appears to be little difference between the conjugated salts, cholate, and dehydrodeoxycholeate, although the latter seems to be somewhat more toxic. There was no significant difference between the effect of the synthetic and natural preparations of the glycocholate and taurocholate. In 4 animals 5 mg. per kilogram of apocholeate produced an average depression of 17 mm. Hg, and deoxycholeate produced 21 mm. Hg. These depressions are greater than those produced by twice the dose of the other four salts in which a 10 mg. dose was tested, and by three times the dose of dehydrocholate. In summary, the salts may be divided into three groups. Group I contains only one salt, dehydrocholate, which is by far the least toxic. Group II contains four salts of intermediate toxicity, cholate, two conjugated salts, and dehydrodeoxycholeate. Finally, Group III contains the two most toxic salts, apocholeate and deoxycholeate.

TABLE I  
DEPRESSOR ACTION OF BILE SALTS

| SALT                | DOSE<br>(MG./KG.) | NO. OF<br>DOGS | BLOOD PRESSURE—MM. OF HG. |         |                       |
|---------------------|-------------------|----------------|---------------------------|---------|-----------------------|
|                     |                   |                | CONTROL                   | MINIMUM | MILLIMETERS<br>CHANGE |
| Dehydrocholate      | 10                | 10             | 144                       | 143     | - 1                   |
| Cholate             | 10                | 10             | 146                       | 138     | - 8                   |
| Glycocholate (N)    | 10                | 10             | 149                       | 138     | -11                   |
| Dehydrodeoxycholate | 10                | 10             | 161                       | 151     | -10                   |
| Apocholate          | 10                | 10             | 152                       | 124     | -28                   |
| Deoxycholate        | 10                | 10             | 158                       | 125     | -33                   |
| Dehydrocholate      | 30                | 10             | 144                       | 134     | -10                   |
| Cholate             | 30                | 10             | 140                       | 112     | -28                   |
| Taurocholate (S)    | 30                | 3              | 119                       | 95      | -24                   |
| Taurocholate (N)    | 30                | 3              | 109                       | 84      | -25                   |
| Glycocholate (S)    | 30                | 3              | 131                       | 103     | -28                   |
|                     |                   | (6 tests)      |                           |         |                       |
| Glycocholate (N)    | 30                | 10             | 135                       | 103     | -32                   |
| Dehydrodeoxycholate | 30                | 10             | 143                       | 102     | -41                   |
| Apocholate          | 30                | 5              | 152                       | 87      | -65                   |
| Deoxycholate        | 30                | 5              | 152                       | 79      | -73                   |
| Apocholate          | 5                 | 4              | 124                       | 107     | -17                   |
| Deoxycholate        | 5                 | 4              | 117                       | 96      | -21                   |

*Comment.*—Selecting cholic acid as the parent compound, the effects of various changes in this basic structure may be examined. Oxidation of the three hydroxyl groups of cholic acid to ketone groups markedly decreases the toxicity. Conjugation of cholic acid with glycine or taurine does not have a marked effect on the toxicity. The removal of the hydroxyl group on C7 of cholic acid very markedly increases toxicity. *Oxidation of the two hydroxyl groups of deoxycholic acid markedly (44 per cent) decreases the toxic properties of the latter compound*, though not so markedly (64 per cent) as the oxidation of cholic acid. Apocholic acid, which differs from deoxycholic only in having one ethylene linkage ( $C8 = C9$ ), has toxic properties similar to those of the saturated substance.

*Hemolysis Studies.*—Difficulty was experienced in obtaining cell suspensions with uniform properties, even though the blood was always taken from the same person and an exactly similar technique was employed in the preparation. However, although the absolute dilutions did not check exactly with different cell suspensions, the order of hemolytic activity of the different salts tested on the same cell suspension was always the same. On the basis of our results the various salts may be divided into three groups according to their hemolytic activity (Table II).

TABLE II  
HEMOLYSIS BY BILE SALTS

|                                |                           |
|--------------------------------|---------------------------|
| Group I —Weakly hemolytic      | 1. Dehydrocholate         |
| Group II —Moderately hemolytic | 1. Synthetic glycocholate |
|                                | 2. Synthetic taurocholate |
|                                | 3. Cholate                |
|                                | 4. Dehydrodeoxycholate    |
| Group III—Strongly hemolytic   | 1. Natural glycocholic    |
|                                | 2. Natural taurocholic    |
|                                | 3. Apocholic              |
|                                | 4. Deoxycholic            |

*Comment.*—Oxidation of the three hydroxyl groups of cholic acid decreases the hemolytic activity of this substance. The removal of the OH group on C7 markedly increases the hemolytic activity. The hemolytic properties of deoxycholic acid are decreased by oxidation to the ketoacid. The conflicting results obtained with the conjugated salts are of interest. The synthetic products were only weakly hemolytic, being second only to the dehydrocholate. On the other hand, the natural products were strongly hemolytic. It is probable that impurities (fatty acids) in the natural products were responsible for the discrepancy. If the synthetic products represent the action of the pure salts, then conjugation does not markedly affect the hemolytic properties of cholic acid.

TABLE III  
CHOLERETIC EFFECTS OF BILE SALTS  
(30 Mg. per kilogram of body weight intravenously)

| SALT                   | NO. OF ANIMALS | BILE VOLUME PER 1 HOUR PERIOD |                            |          | 30 MIN. AFTER INJECTION |
|------------------------|----------------|-------------------------------|----------------------------|----------|-------------------------|
|                        |                | CONTROL*                      | FIRST HOUR AFTER INJECTION | % CHANGE |                         |
| 1. Dehydrocholate      | 5              | 2.0                           | 13.5                       | +575     | 16.8                    |
| 2. Apocholate          | 5              | 2.0                           | 10.0                       | +400     | 11.8                    |
| 3. Deoxycholate        | 6              | 2.0                           | 8.9                        | +345     | 11.2                    |
| 4. Cholate             | 6              | 2.0                           | 7.3                        | +265     | 9.3                     |
| 5. Dehydrodeoxycholate | 5              | 2.0                           | 6.4                        | +220     | 8.3                     |
| 6. Glycocholate (S)†   | 6              | 2.0                           | 6.4                        | +220     | 7.2                     |
| 7. Taurocholate (S)†   | 9              | 2.0                           | 5.5                        | +175     | 6.9                     |

\*In order to illustrate the relative choleretic effects more clearly, all of the volume outputs have been recalculated on the basis of a common control value, i.e., 2 c.c. per hour. The actual control values varied from 1.6 to 2.6 c.c. per hour.

†Sufficient of the natural compound was not available for this study.

The first three salts produced a choleresis lasting somewhat longer than the last four; the choleresis of the last four was completed in one to one and one-half hours.

*Choleretic Studies.*—The results of the choleretic studies are summarized in Table III. Sodium dehydrocholate is by far the best choleretic. It causes a greater and more prolonged choleresis than any of the other salts studied. Based on the percentage increase in bile output during the first hour after injection, the salts may be grouped as follows in the order of decreasing choleretic properties:

|                               |  |
|-------------------------------|--|
| Group I (greatest choleresis) | Dehydrocholate                                     |
| Group II                      | Apocholate<br>Deoxycholate                         |
| Group III                     | Cholate<br>Dehydrodeoxycholate<br>Glycocholate (S) |
| Group IV                      | Taurocholate (S)                                   |

*Comment.*—Oxidation of the three hydroxyl groups of cholic acid to keto groups markedly increases the choleretic properties. It also appears that removal of the hydroxyl group on C7 of cholic acid increases the choleretic activity somewhat. Preliminary results on chronic biliary fistula dogs<sup>15</sup> do not support this finding. In these experiments the bile salts were given orally, and deoxycholic acid proved to be only about two-thirds as effective a choleretic as



eholic acid. On the other hand, it has been reported that in the anesthetized rabbit, apocholic acid produces a greater choleresis than dehydrocholic acid,<sup>13</sup> and that both apocholic and deoxyeholic acid produce a greater choleresis than cholic acid.<sup>10</sup> The results of recent studies<sup>16</sup> suggest that conjugation suppresses the choloretic effect of the ketocholanic acids. The authors of this report point out the fact that their results do not mean that conjugation of all ketocholanic acids will depress their choloretic activity or that the oxidation of all cholanic acids will give them hydrocholoretic properties.

Our results show that equivalent weights of the conjugated acids are less choloretic than cholic acid. Assuming choleresis to be dependent on cholic acid content, and using the choleresis it caused as a base, one would expect approximately a 213 per cent choleresis with taurocholic acid and a 234 per cent choleresis with glycoeholic acid (since our taurocholic acid preparation assayed 80 per cent cholic acid and our glycoeholic 87 per cent cholic acid). Our results thus appear to indicate that conjugation with glycine has little effect on the choloretic properties of cholic acid, whereas conjugation with taurine depresses this property. Preliminary results on chronic fistula dogs also appear to indicate that conjugation with taurine depresses the choloretic action of cholic acid.

It is of interest that oxidation of deoxyeholic acid to the dehydro form did not in our experience increase the choloretic properties of this substance. Adlersberg and Lustig<sup>13</sup> quote unpublished work which indicates that dehydrodeoxyeholic acid is less toxic and more choloretic than the hydroxyacid. They also report acute experiments in rabbits showing that dehydroapocholic acid is more choloretic than apocholic, and that both the latter acids are better choloretics than dehydrocholic acid. Sobotka<sup>17</sup> states that dehydrodeoxyeholic acid is a better choloretic than dehydrocholic. The source of this statement is not given. In our experience, dehydrocholic acid was by far the best choloretic.

#### DISCUSSION

The literature on the toxicity of bile has been completely reviewed in Horrall's recent book.<sup>6</sup> Much of the earlier work is of questionable significance because pure substances were not used. To summarize an extensive literature, toxicity studies by various methods (lethal doses in different animals, effect on excised heart and skeletal muscles, effect on the gall bladder) reveal that cholic acid is much less toxic than deoxyeholic and that dehydrocholic acid is the least toxic of all. The majority of studies on hemolysis place the order of increasing hemolytic activity as follows: dehydroeholic < cholic < apocholic < deoxyeholic. In general, these results are in agreement with our own. It is worthy of note that the relative toxicity, as tested by depression of blood pressure and hemolysis, is practically identical. The structural implications of our results have already been discussed.

The majority of the pertinent literature on choleresis has been cited. Regan and Horrall<sup>11</sup> found glycoeholic acid to be more toxic and less choloretic than dehydrocholic acid. This is in agreement with our findings.

## SUMMARY AND CONCLUSIONS

1. The toxic and choleric properties of seven bile salts have been studied in the dog.

2. Based on depressor and hemolytic activity, dehydrocholate is the least toxic salt, and apocholate and deoxycholate are the most toxic salts. The other four salts form a group of intermediate toxicity.

3. A discrepancy in the hemolytic activity of two different preparations, one synthetic and the other a "natural" but not pure, of the conjugated cholates has been noted. The probable explanation of this fact is discussed.

4. Sodium dehydrocholate, or oxidized cholic acid, or a ketocholic acid, is the most effective choleric, as well as the least toxic. Sodium taurocholate is the least effective. Deoxycholic acid is not a good choleric and is relatively quite toxic.

5. The influence of chemical structure on the toxic and choleric properties of the bile acids has been discussed.

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## MULTIPLE SERUM ALLERGY IN ATOPIC INDIVIDUALS\*

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WITH the increasing use of animal sera in modern therapeutics, the subject of serum allergy is assuming ever increasing importance. It is not the purpose of this paper to undertake a general discussion of this subject,<sup>†</sup> but rather to determine the frequency with which multiple serum allergy occurs in allergic individuals, and the possible dangers involved when serum of one animal is substituted for that of another without preliminary tests.

Serum allergy may be defined as a state of hypersensitivity to a foreign serum, usually the result of a previous serum injection. In the allergic patient it may be coincident with a sensitiveness to the animal dander which contains also a small amount of serum antigen. The parenteral administration of a foreign serum in atopic persons even in minute quantities, is likely to result in serum reactions. The latter may be of the delayed type—accelerated serum sickness, or the precipitating or immediate reaction. A history of asthmatic attacks when in contact with horses, "horse asthma," or, for that matter, a history of allergic manifestations when in contact with other animals, increases the possibility of severe or even fatal reactions to a much greater degree. Boughton<sup>1</sup> in 1919 reported a death which resulted from the administration of one minim of horse serum intravenously to a horse asthmatic. Fatal reaction from an intracutaneous test with 0.05 c.c. of horse serum was described by Friedman<sup>2</sup> in 1935. Lobo<sup>3</sup> in 1936 reported a death which resulted from the subcutaneous injection of 5 c.c. of antidysentery serum to a 1-year-old child with "exudative diathesis." In 1937 Vaughan and Pipes,<sup>4</sup> in reviewing the literature from 1923 to 1935, found reports of 35 fatalities from serum administrations, 6 of the deaths following intracutaneous tests with undiluted serum. Shoek following an intravenous test<sup>7,†</sup> with a 1:50 dilution of rabbit serum in two allergic individuals was described by Horsfall and his co-workers.<sup>5</sup>

As the reports in the literature deal mostly with horse serum, the question arises whether the dangers involved in its use in allergic persons may be circumvented by substituting a serum obtained from an animal other than the horse. Horsfall and his co-workers,<sup>5-7</sup> for example, have advocated the substitution of antipneumococcus serum obtained from the rabbit for that of the horse. They set forth the many desirable advantages that it offers immunologically and also

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†For a comprehensive analysis of the literature on serum allergy, the reader is referred to a review by Harten and Walzer, *J. Allergy* 11: 68, 1939.

‡These authors feel that the intravenous test to establish serum sensitivity in allergic persons, because of the possible occurrence of severe immediate serum reactions or even fatalities, is a procedure fraught with much danger and should, therefore, not be used routinely, if at all.

pointed out that a smaller percentage of persons are apt to be sensitive to rabbit serum than to horse serum. That such is the case cannot be denied, considering the fact that immune sera up to recent times have been derived from the horse almost exclusively. One must bear in mind, however, that the occurrence of atopic serum allergy does not necessarily require a previous serum injection, as exposure to the animal dander in allergic persons may sensitize them to the homologous serum as well. That horse dander contains also a small amount of horse serum antigen has been amply demonstrated by the work of Raekemann,<sup>8</sup> Forster,<sup>9</sup> Ratner and Gruehl,<sup>10</sup> and Tuft.<sup>11</sup> That this is also true of other animal danders may be deduced from the work of Walker,<sup>12</sup> who showed that 70 per cent of his cat dander cases were also allergic to cat serum. These figures indicate an even higher percentage of serum antigen in the danders of these animals than in that of the horse, since he obtained only 22 per cent of horse serum reactions in those allergic to the horse dander.

Simon<sup>13</sup> in 1934 reported 12 patients whom he skin-tested with 9 mammalian sera and found that 3 of these reacted to all 9 sera, 4 reacted to horse serum plus one or two additional sera, and 5 were sensitive to horse serum alone. As a result of these findings, he concluded that there must be a common serum antigen in all mammalian sera in addition to a species-specific antigen characteristic of each one; and that whereas most people become sensitized to the latter factor, some few become allergic to the common factor. Two of 67 patients with pneumonia reported by Horsfall and his co-workers<sup>7</sup> were found to be sensitive to horse serum and went into shock after an intravenous test with rabbit serum, thus indicating that they were sensitive to at least two sera.

It appears, therefore, from these observations that there are persons who may be sensitive to more than one serum. Indeed, some probably are sensitive to all mammalian sera. In view of these facts it was our object to investigate: (1) the frequency with which sensitivity occurs in allergic persons to the serum of horse and other animals; (2) the value of the skin and ophthalmic tests in the diagnosis of serum allergy; (3) the relationship between serum and dander allergy; and (4) the comparative incidence of horse serum allergy in allergic persons who previously had received serum injections and in those who had not.

Forty patients from our Allergy Clinic, consisting of hay fever and asthma cases, were tested with 1:100 and 1:10 dilutions of horse, goat,\* rabbit,\* and sheep serum with the intracutaneous skin and ophthalmic tests, and also with the corresponding danders (Table I). Of these, 3 cases originally reacted to 1:100 dilution of horse serum by the intracutaneous skin test with a four-plus reaction. These cases and any others that reacted to the higher dilution of a serum were, for obvious reasons, not tested with the 1:10 dilution. If the reaction with the 1:100 dilution was three plus or less, a 1:10 dilution of the +

\*Rabbit and goat sera were kindly supplied by the Lederle Laboratories.

†The reactions were read after an interval of ten to fifteen minutes, according to the following scheme:

|  |             |
|--|-------------|
| Four plus—large wheal 2 cm. in diameter or           | seudopodia. |
| Three plus—wheal smaller than above, with no         |             |
| Two plus—definite wheal smaller than above, 2.       |             |
| One plus—erythematous flare with slight or no wheal. |             |
| 0—no discernible reaction.                           |             |

different sera was used for skin testing. On a subsequent visit,\* conjunctival tests† were performed with 1:100 dilution of the sera. If the test was positive, the higher concentration was not used. When the opposite was the case, the tests were repeated with the 1:10 dilutions. No more than one conjunctival test was done at one time; the other eye was used as a control. In case of obvious lack of sensitivity more than one serum was used for testing after a proper interval.

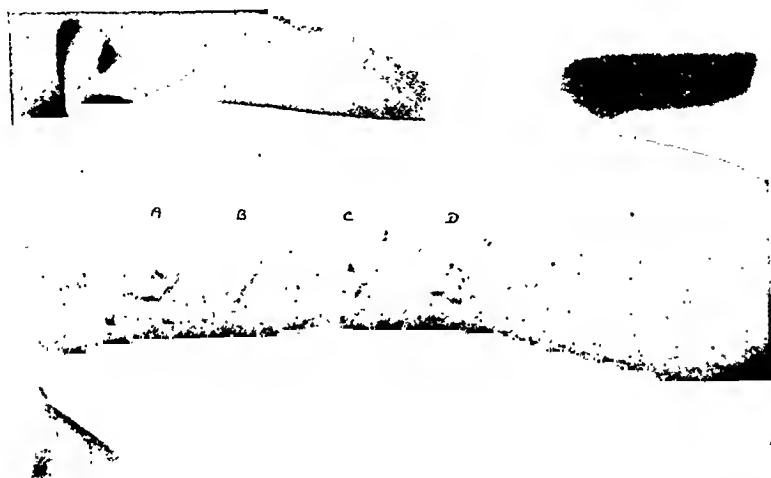


Fig. 1.—Case 1. Patient S. B. Direct intracutaneous skin tests with 4 different sera diluted 1:100. Photographed after twenty minutes. All reactions were markedly positive to the 4 sera, showing wheal with pseudopods and area of erythema. *a*, Horse serum 1:100; *b*, goat serum 1:100; *c*, rabbit serum 1:100; *d*, sheep serum 1:100; *e*, control, buffered saline; *f*, control, buffered saline.

Of the 40 allergic individuals studied, 3, or 7.5 per cent, yielded four-plus skin and positive conjunctival reactions to all 4 sera (Table II; Figs. 1 and 2). These 3 cases in addition exhibited systemic reactions besides the marked local reactions. The former consisted of sneezing fits and attacks of asthma which required the administration of epinephrine. One patient (Case 1) also exhibited angioneurotic edema of eyelids and face, and generalized urticaria; another (Case 24) showed four-plus skin reactions to goat and sheep sera, and negative reactions to the other two sera; the ophthalmic reactions were not done due to the patient's failure to return to the clinic. Four patients, or 10 per cent, evidenced four-plus skin reactions to one or more serum, 3 of them also exhibited positive conjunctival tests, 1 was not tested; the remaining 36 cases evidenced skin reactions varying from three to one plus; and none gave a positive eye test. If all degrees of skin reactions are considered, then there were 25 cases who reacted to horse serum, 21 to goat serum, 21 to rabbit serum, and 22 to sheep serum. Completely negative reactions were obtained in the remainder (Table II). The number of four-plus skin reactions obtained by Brown and Seelzer<sup>14</sup>

\*It was found wise to perform the intracutaneous and ophthalmic tests on different days, since positive reactions to both were followed by constitutional reactions in 3 cases, necessitating the use of adrenalin, and, in one case, the use of aminophyllin also.

†A conjunctival test was considered positive if there occurred a definite redness of the palpebral and bulbar conjunctivae due to the injection of the conjunctival vessels associated with itching and lacrimation within five to ten minutes after the instillation of the test solution into the conjunctival sac. In case of marked reactions systemic manifestations also occurred.

TABLE

## RESULTS OF INTRACUTANEOUS AND OPHTHALMIC

| CASE      | AGE | SEX | ALLERGY<br>ON<br>CONTACT<br>WITH<br>ANIMALS | HISTORY OF<br>PREVIOUS<br>SERUM INJECTIONS | HISTORY<br>OF<br>PREVIOUS<br>SERUM<br>SICKNESS | HORSE               |      |                 |      |        |     |
|-----------|-----|-----|---|--|--|---------------------|------|-----------------|------|--------|-----|
|           |     |     |   |  |  | SERUM               |      |                 |      | DANDER |     |
|           |     |     |   |  |  | INTRACU-<br>TANEOUS |      | OPHTHAL-<br>MIC |      |        |     |
|           |     |     |   |  |  | 1-100               | 1-10 | 1-100           | 1-10 | 100    | 10  |
| 1.—S. B.  | 20  | F   | No  | D.A.T.*                                    | No   | +++                 |      | +++             |      | +++    |     |
| 2.—N. B.  | 9½  | F   | No  | No   | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 3.—N. B.  | 9½  | M   | No  | T.A.T.†                                    | No   | 0                   | ++   | 0               | 0    | 0      | 0   |
| 4.—M. C.  | 50  | F   | No  | No   | No   | 0                   | 0    | 0               | 0    | ++     | +++ |
| 5.—R. D.  | 32  | F   | No  | D.A.T. at age<br>of 12                     | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 6.—I. E.  | 30  | M   | No  | T.A.T.                                     | No   | 0                   | +    | 0               | 0    | 0      | 0   |
| 7.—N. E.  | 12  | M   | No  | No   | No   | 0                   | 0    | 0               | 0    | +      | +   |
| 8.—W. G.  | 22  | M   | B.A.‡<br>Rabbit<br>wool?                    | No   | No   | +++                 |      | +++             |      |        | ++  |
| 9.—H. G.  | 13  | F   | No  | D.T.A.T.‡                                  | No   | ++                  | +++  | 0               | 0    | 0      | 0   |
| 10.—R. K. | 30  | M   | No  | D.T.A.T.—twice<br>last 18 mo.              | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 11.—Y. K. | 15  | F   | No  | T.A.T.—twice<br>2 and 3 yr. ago            | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 12.—F. L. | 34  | F   | No  | No   | No   | 0                   | ++   | 0               | 0    | 0      | +   |
| 13.—L. M. | 42  | F   | No  | No   | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 14.—C. M. | 56  | F   | No  | No   | No   | ++                  | ++   | 0               | 0    | ++     | +++ |
| 15.—S. P. | 14  | F   | No  | No   | No   | +++                 |      | +++             |      | +++    |     |
| 16.—R. P. | 6   | M   | No  | No   | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 17.—J. S. | 41  | F   | No  | No   | No   | +                   | +    | 0               | 0    | 0      | 0   |
| 18.—I. I. | 42  | F   | No  | No   | No   | 0                   | +    | 0               | 0    | 0      | +   |
| 19.—L. W. | 14  | M   | No  | D.T.A.T.                                   | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 20.—M. W. | 41  | M   | No  | No   | No   | 0                   | +    | 0               | 0    | 0      | 0   |
| 21.—M. Z. | 55  | F   | B.A.‡<br>Horses                             | No   | No   | +                   | +    | 0               | 0    | +++    | 0   |
| 22.—A. P. | 51  | M   | No  | No   | No   | +                   | +    | 0               | 0    | 0      | 0   |
| 23.—M. H. | 50  | M   | No  | No   | No   | 0                   | +    | 0               | 0    | 0      | 0   |
| 24.—P. G. | 32  | F   | No  | No   | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 25.—J. W. | 28  | M   | No  | No   | No   | +                   | +    | 0               | 0    | 0      | ++  |
| 26.—B. F. | 55  | M   | No  | No   | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 27.—S. F. | 32  | F   | No  | No   | No   | 0                   | +    | 0               | 0    | 0      | 0   |
| 28.—P. G. | 60  | M   | No  | No   | No   | +                   | ++   | 0               | 0    | +      | ++  |
| 29.—A. H. | 53  | M   | No  | No   | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 30.—P. S. | 11  | M   | No  | T.A.T.—twice<br>at age 3                   | No   | ++                  | +++  | 0               | 0    | 0      | 0   |
| 31.—J. C. | 8   | M   | No  | T.A.T.—twice<br>at age 7                   | No   | 0                   | +    | 0               | 0    | 0      | 0   |
| 32.—A. R. | 54  | M   | No  | No   | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 33.—R. B. | 10  | F   | No  | D.T.A.T.                                   | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 34.—I. B. | 40  | M   | No  | No   | No   | 0                   | +    | 0               | 0    | 0      | 0   |
| 35.—A. N. | 54  | M   | No  | No   | No   | 0                   | +    | 0               | 0    | +      | ++  |
| 36.—E. G. | 32  | F   | No  | D.A.T.                                     | No   | 0                   | +++  | +++             |      | +++    |     |
| 37.—P. R. | 33  | F   | No  | No   | No   | 0                   | ++   | 0               | 0    | +      | ++  |
| 38.—F. G. | 36  | F   | No  | No   | No   | +                   | ++   | 0               | 0    | 0      | 0   |
| 39.—S. B. | 38  | M   | No  | No   | No   | 0                   | 0    | 0               | 0    | 0      | 0   |
| 40.—R. F. | 41  | F   | No  | No   | No   | +                   | +    | 0               | 0    | 0      | 0   |

\*D.A.T.—Diphtheria antitoxin.

†T.A.T.—Tetanus antitoxin.

‡B.A.—Attack of bronchial asthma.



with rabbit serum tests in 101 allergic persons were 2 as compared with 3 such reactions in our series of 40 cases; we obtained 3 positive conjunctival tests to their 1. The discrepancy in these figures may be explained by the fact that these authors tested 101 cases at random, whereas we started with 3 patients who were allergic to horse serum.



Fig. 2.—Case 1. Patient S. B. Positive ophthalmic test of right eye (one drop of 1:100 sheep serum was instilled); left eye control. Photographed after ten minutes. Reaction, however, started in about three minutes. Patient experienced marked itching and also tearing of the eye. Rhinorrhea, sneezing, and choking of the chest with dyspnea also occurred. Adrenalin 1:1,000 locally and subcutaneously had to be administered.

Brief case histories of 5 persons who reacted positively to one or more serum and one or more danders are given.

#### CASE REPORTS

CASE 1.—S. B., a female stenographer, aged 20 years, had perennial bronchial asthma. She gave no history of allergic symptoms on contact with animals, nor of any previous serum administration. She reacted to all 4 sera both by skin and eye tests. Four-plus skin reactions were also obtained with horse and cat dander and pork, beef, and lamb. There were clinical improvements from the elimination of these foods. She exhibited constitutional reactions following skin tests with all 4 sera, conjunctival test with one of them necessitating the use of adrenalin and aminophyllin for the relief of asthma and angioneurotic edema (Figs. 1 and 2).

CASE 8.—W. G., a male, aged 22 years, waiter at the Hospital, had perennial asthma. He gave a definite history of asthmatic attacks when in proximity to rabbits and when working in a sweater factory. There was no history of any serum injections. He reacted to all 4 sera both by skin and conjunctival tests, and also showed four-plus skin reactions to goat, rabbit, cat and dog epithelia, and also to pork, lamb, beef, and milk. Constitutional reaction resulted from the positive skin and eye tests, necessitating the use of adrenalin.

CASE 15.—S. P., a 14-year-old schoolgirl, had perennial asthma. She had had diphtheria toxin-antitoxin in childhood, but no other sera. She gave no history of any allergic manifestations on contact with animals. Four-plus skin reactions to all 4 sera, as well as positive conjunctival tests, were obtained. The danders of horse, rabbit, cat, and dog, as well as pork, lamb, and beef, yielded four-plus skin reactions. Constitutional reaction resulted from the positive skin and eye tests, necessitating the use of adrenalin.

CASE 24.—P. G., an Austrian female, aged 32 years, domestic, had seasonal hay fever and was asthma sensitive to grasses. She presented no history of any prior serum administration



nor allergy on contact with animals. Goat and sheep sera yielded positive four-plus skin reactions. Horse and rabbit sera yielded negative reactions. The ophthalmic reactions were not done since the patient failed to return. No reactions to the homologous or to other danders were obtained.

CASE 36.—E. G., a female, aged 32 years, housewife, had seasonal hay fever and was asthma sensitive to ragweed and trees. She had diphtheria antitoxin in childhood, but no other sera. She gave no history of allergic symptoms on contact with animals. She had a four-plus skin and positive conjunctival test to horse serum. She did not react to the other sera. She also exhibited a four-plus skin reaction to horse dander but not to other danders.

In studying the case histories of patients who showed positive reactions to serum, it may be noted that they also reacted to one or more animal danders. It has already been mentioned earlier in this paper that animal danders also contain a small amount of the homologous serum antigen. Exposure, therefore, to the animal dander may well sensitize an allergic individual to the serum. This may serve to explain, as brought out by Ratner and Gruehl's<sup>20</sup> experiments on guinea pigs, the existence of allergy to serum in atopic persons in the absence of previous serum injections. Of the 3 patients who evidenced four-plus skin and positive eye reactions to all 4 sera, one (Case 8) reacted with four-plus skin reactions also to goat and rabbit danders; another one (Case 1) evidenced similar reactions to horse dander; another (Case 15) to horse and rabbit danders. The one (Case 24) that reacted only to goat and sheep sera did not exhibit any positive reactions to dander, and the one (Case 36) who was positive to horse serum only also reacted to horse dander. Although the number of cases with positive dander reactions\* (4 plus) are too small in this series to warrant arriving at any conclusions, it may nevertheless be of interest to note that in atopic persons who are sensitive to animal danders, there is a tendency for them to be also allergic to the homologous serum. The 4 patients with four-plus reaction to horse epithelium were also positive to the homologous serum by skin and eye tests; one case out of 2 with four-plus reaction to the goat dander was also allergic to the serum; both cases with four-plus reaction to rabbit dander also reacted to the serum. Not one out of 4 positive sheep dander cases reacted to the serum.

Tuft<sup>15</sup> in 1932 in a study of serum allergy after diphtheria toxin-antitoxin administration found that those who gave positive reactions showed a positive family or personal history of allergy in 34 per cent of cases, as compared with 7 per cent in controls. These findings, he states, show that the "allergic constitution is an important factor in predisposing these individuals to acquired serum allergy." He also offers evidence to prove that this may not be mediated through the reagin mechanism. In our group of 40 allergies, as far as could be determined by questioning, only 12 had previously received either diphtheria toxin-antitoxin, or diphtheria or tetanus antitoxin (Table III). Two of this group showed four-plus skin and positive conjunctival reactions to horse serum. Two of the 28 who never had received any serum yielded similar reactions. If all degrees of positive reactions to horse serum are included, then 7, or 58.3 per

\*Tests were performed with 0.001 and 0.01 concentrations of dander extract (milligrams of nitrogen per cubic centimeter). In case of four-plus reaction to the former, higher concentration was not tested.

cent, of the 12 cases who previously had received serum, reacted to horse serum, and 18, or 64.5 per cent, of those who had not had any serum previously reacted to it. It appears, therefore, that there is very little difference in reactivity in these two groups. Since the series is small, no conclusions are attempted.

TABLE II

FORTY ALLERGICS TESTED WITH FOUR MAMMALIAN SERA: HORSE, GOAT, RABBIT, SHEEP

|                 | HORSE SERUM                                      |    |    |    |    | GOAT SERUM |    |    |    |    | RABBIT SERUM |    |    |    |    | SHEEP SERUM |    |    |    |    |
|-----------------|--|----|----|----|----|------------|----|----|----|----|--------------|----|----|----|----|-------------|----|----|----|----|
|                 | REACTIONS  |    |    |    |    |            |    |    |    |    |              |    |    |    |    |             |    |    |    |    |
|                 | 4+   | 3+ | 2+ | 1+ | 0  | 4+         | 3+ | 2+ | 1+ | 0  | 4+           | 3+ | 2+ | 1+ | 0  | 4+          | 3+ | 2+ | 1+ | 0  |
|                 | <i>Intracutaneous Tests Using 1:10 Dilutions</i> |    |    |    |    |            |    |    |    |    |              |    |    |    |    |             |    |    |    |    |
| Number of cases | 4  | 2  | 6  | 13 | 15 | 4          | 0  | 3  | 14 | 19 | 3            | 0  | 6  | 12 | 19 | 4           | 0  | 8  | 10 | 18 |
|                 | <i>Conjunctival Tests Using 1:10 Dilutions</i>   |    |    |    |    |            |    |    |    |    |              |    |    |    |    |             |    |    |    |    |
| Number of cases | 4  | 0  | 0  | 0  | 36 | 3          | 0  | 0  | 0  | 37 | 3            | 0  | 0  | 0  | 37 | 3           | 0  | 0  | 0  | 37 |

TABLE III

COMPARATIVE INCIDENCE OF POSITIVE SKIN AND CONJUNCTIVAL TESTS IN ALLERGICS WHO HAD PREVIOUSLY RECEIVED HORSE SERUM INJECTIONS WITH THOSE WHO HAD NOT

|   | NUMBER OF CASES | REACTIONS                         |                                   |                                   |                                   |  | NEGATIVE SKIN AND CONJUNCTIVAL |
|---|-----------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|--|--------------------------------|
|   |                 | 4+ SKIN AND POSITIVE CONJUNCTIVAL | 3+ SKIN AND NEGATIVE CONJUNCTIVAL | 2+ SKIN AND NEGATIVE CONJUNCTIVAL | 1+ SKIN AND NEGATIVE CONJUNCTIVAL |  |                                |
| Those who previously had horse serum injections | 12              | 2                                 | 2                                 | 1                                 | 2                                 |  | 5                              |
| Those who never had any serum injections        | 28              | 2                                 | 0                                 | 4                                 | 12                                |  | 10                             |

## SUMMARY

1. Four-plus skin and positive conjunctival tests were considered in this study as indicating allergy to serum. On the basis of the 40 allergies tested, 5, or 12.5 per cent, exhibited positive reactions to one or more sera; 4, or 10 per cent, to two or more sera; and 3, or 7.5 per cent, to all 4 sera. If all degrees of reaction are included, then 25 were completely negative to horse serum; 19 to goat and rabbit sera, and 18 to sheep serum.

2. Every case with a four-plus skin reaction to a serum also exhibited a positive conjunctival test, and vice versa. At no time was a positive conjunctival test obtained where the intracutaneous test to the serum was less than four plus.

3. a. Correlation between positive reactions to serum, and the corresponding dander antigen:

- (1) Of the 3 cases that reacted to the 4 sera, one case also reacted to horse dander; one to goat and rabbit dander, and one to horse and rabbit.
- (2) The case that reacted to goat and sheep serum evidenced no positive skin reactions to any danders.
- (3) The case that reacted to horse serum alone also was positive to the corresponding dander.

b. correlation between positive reactions to animal danders, and the corresponding sera:

- (1) Of 4 cases that reacted with a four-plus reaction to horse dander, all 4 also were positive by skin and conjunctival tests to the homologous serum.
- (2) Of 2 cases that evidenced four-plus reaction to goat dander, one reacted to goat serum by skin and eye tests.
- (3) Both cases that reacted with a four-plus reaction to rabbit dander also showed positive skin and eye tests to the serum.
- (4) None of the 4 cases that reacted to sheep dander reacted to the serum. This may indicate that reaction to sheep dander is not very specific, or that there is a relatively low degree of association of sheep dander and sheep serum allergy.
- (5) Twenty-eight, or 70 per cent, of the cases had never received any serum injections, while 12, or 30 per cent, did. There was very little difference noted in the number of positive reactions to horse serum in the two groups. Moreover, there were just as many positive reactions to goat, rabbit, and sheep sera where the question of prior serum injection was not a factor.

#### CONCLUSIONS

1. Atopic persons may be sensitive to the sera of one or more species of animals.

2. A four-plus skin and conjunctival reaction to a serum should be considered *prima facie* evidence that sensitivity to it exists, and its avoidance or the extreme care in its use is indicated.

3. Sensitivity to an animal dander is coincident in many cases with a sensitivity to the corresponding serum.

4. In our series we found very little difference in the frequency of positive reactions to horse serum in allergic persons who previously had had serum injected (diphtheria and tetanus antitoxin and diphtheria toxin-antitoxin), and in those who had never received any serum. Extreme care should be exercised in the use of therapeutic sera in any allergic patient.

5. In all cases the use of immune sera obtained from any species of animal is not devoid of the possibility of serum reactions. In all circumstances skin and conjunctival tests should be performed before a serum from any animal is injected. The presence of a conjunctival reaction is a danger signal which should not be ignored.

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# CLINICAL CHEMISTRY

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## PROTHROMBIN STUDIES USING RUSSELL VIPER VENOM\*

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### II. RELATION OF CLOTTING TIME TO PROTHROMBIN CONCENTRATION IN HUMAN PLASMA

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THE use of Russell viper venom instead of tissue extract as the source of thromboplastic substance in determining the prothrombin clotting time was reported first by Fullerton,<sup>1</sup> and later by Page and Russell.<sup>2</sup>

The end point of the test is taken as the number of seconds required for a fibrin web to form when calcium is added to a mixture of oxalated plasma and Russell viper venom. The time (result in seconds) obtained is generally considered to be inversely proportional to the concentration of prothrombin in the plasma. In order to determine the relation of prothrombin concentration to clotting time, using Russell viper venom as the thromboplastic agent, the study outlined here was carried out.

#### METHOD OF STUDY

Blood of normal persons and of patients attending an outpatient clinic is obtained. Only blood from patients not suspected of having liver damage or a vitamin K deficiency is used. Four and one-half cubic centimeters of venous blood are drawn into a dry syringe and mixed with 10 mg. of potassium oxalate in a centrifuge tube. The blood is centrifuged at 1,500 r.p.m. for five minutes and the oxalated plasma is drawn off. The test is performed as follows: 0.2 c.c. of oxalated plasma is pipetted into a small test tube (75 by 10 mm.) and 0.2 c.c. of Russell viper venom,† 1:10,000 solution, is added. Calcium chloride solution (1.11 Gm. calcium chloride per 100 c.c.), 0.2 c.c., is then added, and the stop watch is started. The tube is agitated for ten to fifteen seconds in a water bath (37.5° C.), then removed and tilted until separate discrete fibrin particles can be seen. The first appearance of the fibrin particles is taken as the end point. The prothrombin clotting time is determined on the undiluted plasma (100 per cent). Then a sample of the 100 per cent plasma is diluted with physiologic saline solution so that it represents 80 per cent plasma, and its prothrombin clotting time is determined. Serial dilutions are made containing,

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†Supplied as 'Stypven' Russell viper venom by Burroughs Wellcome & Co., Inc., New York, N. Y.

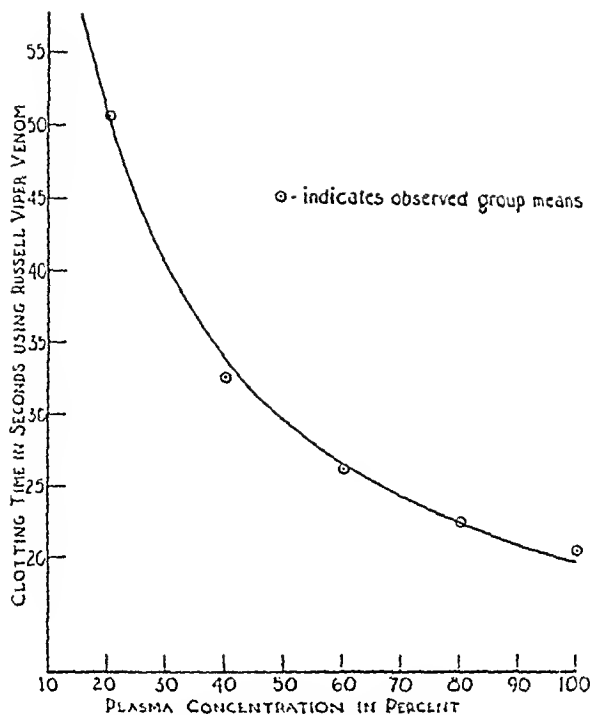


Fig. 1.—Showing the agreement between the plotted points and the curve calculated from the equation  $T = 287.3X^{-0.201}$ , where  $T$  is the clotting time in seconds and  $X$  is the percentage concentration of the plasma. Each plotted point is an average of 26 values.

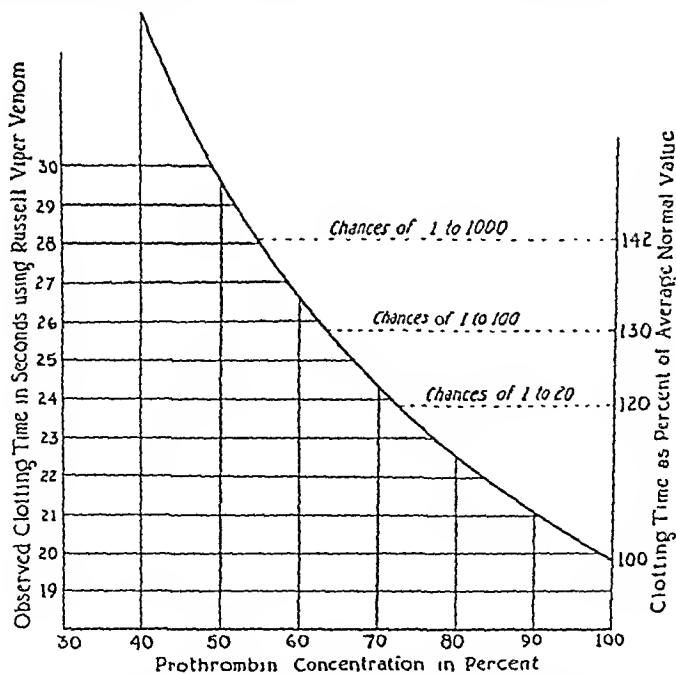


Fig. 2.—A chart giving the probability that an observed prothrombin clotting time or prothrombin concentration is normal. Case I. When the clotting time has been determined by the method described in this paper, or by some type of Quick's method giving essentially the same results (Page and Russell<sup>2</sup>), use the left-hand scale and then locate the corresponding point horizontally opposite on the curve. The nearest probability may then be read directly. Case II. When the average normal value has been found by experience to be appreciably different from twenty seconds, express the observed clotting time as a per cent of the average normal value and use the righthand scale. For example, if the average normal value in the experience of the laboratory has been found to be 22.2, and the particular observed value to be 30 (a figure which is about 135 per cent of the normal), the right-hand scale shows that the chances of this value being normal are quite small, i.e., between 1:100 and 1:1,000.

respectively, 60, 40, and 20 per cent of plasma, and the prothrombin clotting time is determined for each dilution. Each test is performed in triplicate, and the average time is taken for the result.

If the oxalated blood is allowed to stand for more than two hours before centrifugation, the prothrombin clotting time is usually increased, but occasionally it is decreased, the latter probably due to hemolysis with release of lecithin. If, on the other hand, the oxalated blood is centrifuged immediately, or at least within an hour, and the separated plasma is allowed to stand at room temperature for one and one-half to five hours, the prothrombin clotting time is usually increased in both the 100 per cent and 20 per cent plasma dilutions. These changes in prothrombin clotting time incident to standing led us to perform the test as soon as possible after the withdrawal of the blood.

#### RESULTS OF STUDY

The routine was carried out on 26 persons, and the resulting data are summarized in Table I. By extensive statistical treatment, the hyperbola of Fig. 1 was found to fit these data. Quick<sup>3, 4</sup> reports similar findings for data obtained by his methods.

TABLE I

PROTHROMBIN CLOTTING TIME DATA FOR DIFFERENT CONCENTRATIONS OF PLASMA USING SAMPLES OF BLOOD FROM 26 PATIENTS

|                    | PERCENTAGE |       |       |       |       |
|--------------------|------------|-------|-------|-------|-------|
|                    | 100        | 80    | 60    | 40    | 20    |
| Mean               | 20.76 sec. | 22.81 | 26.60 | 33.10 | 51.36 |
| Maximum            | 25.0       | 27.8  | 33.4  | 42.2  | 69.5  |
| Minimum            | 15.4       | 16.5  | 17.0  | 20.0  | 40.4  |
| Standard deviation | 2.32       | 2.99  | 3.80  | 5.82  | 8.67  |

#### DISCUSSION

What is normal prothrombin clotting time? The question as to whether a particular clotting time is normal or abnormal is of great practical importance, and because of individual variations is difficult to answer. However, by means of Fig. 2\* it is possible to estimate what the chances may be that a given high clotting time represents a high normal figure. If the chances are very slight that the high figure is normal, one may then be more certain that the high value is due to abnormal causes. Thus for example, if the clotting time of a patient's plasma is twenty-four seconds (corresponding to a plasma prothrombin concentration of about 70 per cent) should the value be considered a high normal, or does it indicate an abnormal clotting time? Reference to Fig. 2 reveals that the chances of such a clotting time being normal are only 1 to 20, and that the value must be considered on the border line. If the clotting time is twenty-six seconds corresponding to a plasma prothrombin concentration of about 63 per cent, the figure may quite safely be considered abnormal, since the chances are

\*Fig. 2 was constructed as follows: All clotting times were expressed as logarithms, since the distribution then appears to be more nearly normal, and the expected mean and the standard deviation were found to be 1.2961 and 0.04955, respectively. Three multiples of the standard deviation (0.0815, 0.1153, and 0.1531) corresponding to the probabilities 0.05, 0.01, and 0.001 were added to the mean. The antilogs of these three sums were found, and the resulting clotting times were marked off on the curve of Fig. 2. The three factors for multiplying the standard deviation may be computed from such tables as Pearl's areas of the normal probability curve or the probit tables of Bliss.<sup>4</sup>

1 to 100 against a normal clotting time being so high. If the clotting time is twenty-eight seconds, the plasma prothrombin concentration is only about 55 per cent, and the certainty that an abnormal condition exists is even greater, since the chances that it might be normal have now dropped to 1:1,000.

It is recognized that the same method of prothrombin determination in the hands of different workers does not always yield identical results. Since insufficient evidence exists as to how close an agreement may be expected between different laboratories, Fig. 2 has been made sufficiently adaptable to take care of a variety of conditions. When the average clotting time differs appreciably from the value reported in this paper, an adjustment may readily be made by expressing the clotting time as per cent of the mean.

Differences in the standard deviation which measures the variability about the mean must also be considered. Table I shows that the unselected data of this study are quite closely grouped about a mean value of about twenty seconds. If the data in question are much more scattered than these, the deviation of any suspected observation from the average normal value must be relatively greater before it can be considered abnormal. To take an extreme case, suppose that the standard deviation is twice as great as that reported in this paper. Then the three clotting times corresponding to chances of 1:20, 1:100, and 1:1,000, would have increased only to twenty-nine, thirty-four, and forty seconds, respectively. If, on the other hand, the standard deviation is less, then smaller deviations of the clotting time from normal would be significant.

#### SUMMARY

Plasma prothrombin dilution studies, using a modified Quick's method employing Russell viper venom instead of brain substance extract, have been carried out on 26 human beings who were found to be normal by clinical tests.

In our hands, using normal undiluted (100 per cent) human plasma, this test gave an average prothrombin clotting time of 20.6 seconds.

When the normal plasma was diluted with physiologic saline according to the following proportions, the prothrombin clotting time increased as the dilution was increased.

| OXALATED PLASMA<br>PER CENT | MEAN PROTHROMBIN CLOTTING TIME<br>SECONDS |
|-----------------------------|---|
| 100                         | 20.76                                     |
| 80                          | 22.81                                     |
| 60                          | 26.60                                     |
| 40                          | 33.10                                     |
| 20                          | 51.36                                     |

Graphs for computing the plasma prothrombin concentration (in terms of percentage of normal) from a known clotting time were worked out and are presented in Figs. 1 and 2.

The probability of an increased prothrombin clotting time being normal was given consideration. If the clotting time increases, the odds that it could still be normal decrease, and a statistical computation of this part of the study has been incorporated into a graph that may be used in connection with any method for determining prothrombin clotting time.

We wish to express our appreciation for the assistance and cooperation of Dr. Louis Bauman in whose laboratory this work was carried out.



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## THE SIGNIFICANCE OF THE XANTHOPROTEIN REACTION IN RENAL INSUFFICIENCY AND OTHER CLINICAL CONDITIONS\*

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IN 1939 Kenny and Hubbard<sup>1</sup> reviewed the literature and reported their results based on 46 xanthoprotein determinations performed on 39 patients with renal insufficiency. Among other statements, they concluded that "aside from acute nephritis, the xanthoprotein reaction offers the best index of uremic intoxication and that uremic symptoms in individual cases may not parallel retention of urea nitrogen but rather that of aromatic substances."

The differential diagnosis between uremia and other clinical conditions simulating the uremic state is not always easy, especially so when a patient is seen for the first time in coma or in a comatose state. This is particularly true at the Cook County Hospital where numerous patients in whom early diagnosis is imperative, are brought into the emergency room. Although the blood non-protein nitrogen and creatinine levels are routinely determined on all hospitalized patients, these are time-consuming procedures and not infrequently, therefore, accurate diagnosis must await these laboratory reports.

Impressed by this report and an earlier one by Steen<sup>2</sup> in which he analyzed 210 tests on blood and body fluids from living patients and from autopsies, we have studied the xanthoprotein reaction in renal insufficiency (uremia) and other clinical conditions in order to determine its efficacy in diagnosis and prognosis in uremia.

In 1924 Becher and his associates<sup>3</sup> reported an increase of aromatic substances—an ether-soluble group comprising phenols, cresols, aromatic oxyacids and indoxyl, and a nonether soluble group containing the amino acids, tyrosine, phenylalanine, and tryptophane—in the blood of patients with renal insufficiency. In 1925 Becher and Koch<sup>4</sup> reported markedly increased xanthoprotein values (80 to 250) in patients with uremia and indicated that uremia was less dependent on retention of nitrogenous products than on that of aromatic sub-

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stances, and that phenol poisoning resembled true uremia. Irdelp, Guchan, and Kazin<sup>5</sup> placed the normal xanthoprotein value between 15 and 35 and showed increased values in all types of renal insufficiency.

In our study we have performed 354 xanthoprotein determinations on 295 patients with various clinical conditions, according to the following technique, which is slightly modified from that originally described by Beeher:<sup>6</sup>

To 3 or 4 c.c. of oxalated blood or serum, add an equal volume of 20 per cent trichloroacetic acid and mix thoroughly. Allow to stand for several minutes and filter through a Whatman No. 40 quantitative filter paper. The filtrate must be absolutely clear, since a slight turbidity will obviate the result. If the filtrate is turbid, refilter it using a little pumice or talcum to clarify. To 1 c.c. of the clear filtrate add 1 drop of concentrated sulfuric acid, 0.5 c.c. of concentrated nitric acid, and a glass bead, and boil over a free flame for about a minute. Cool and add drop by drop about 1 c.c. of saturated sodium hydroxide till the mixture is alkaline. A marked golden color is obtained when phenolic bodies are present. A pale straw color is considered a negative test.

*Quantitative Expression.*—Make all measurements accurately and boil as evenly as possible so as to avoid excessive evaporation. Compare the color of the final mixture with a standard 0.3874 per cent solution of potassium dichromate. Prepare a series of standards as shown in Table I.

TABLE I

| NO. | K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub><br>0.3874% | WATER    | DILUTION | XANTHOPROTEIN<br>NUMBER |
|-----|--|----------|----------|-------------------------|
| I   | 0.1 c.c.   | 0.9 c.c. | 1:10     | 100                     |
| II  | 0.1 c.c.   | 1.9 c.c. | 1:20     | 50                      |
| III | 0.1 c.c.   | 2.9 c.c. | 1:30     | 33½                     |
| IV  | 0.1 c.c.   | 3.9 c.c. | 1:40     | 25                      |
| V   | 0.1 c.c.   | 4.9 c.c. | 1:50     | 20                      |

Compare the unknown in the tube macroscopically with the diluted standards or in a colorimeter. Thus, if the unknown solution matches standard No. II, the xanthoprotein number is 50; the colorimetric readings would be

$$\frac{\text{Standard set at 10}}{\text{Unknown reads 19.5}} \times 0.1 \times 1,000 = 50+.$$

Since normal equals 15-35, macroscopic comparison was considered sufficiently accurate.

## RESULTS

*Renal Insufficiency (Uremia).*—Sixty-four xanthoprotein determinations were performed on 36 patients with uremia. Of these, 34 showed a rise in xanthoprotein values above 60, associated with a uremic rise in nitrogenous retention (i.e., 90 mg. nonprotein nitrogen per 100 c.c. of blood or higher), although the rise in the xanthoprotein reaction did not always correspond in degree to the rise in the blood nonprotein nitrogen level.

Xanthoprotein values ranged from 62 to 460, and the nitrogenous retention from 37 to 250 mg. per 100 c.c. In two cases the xanthoprotein values were below 60, while the blood nitrogenous level was above 90, i.e., of uremic

nature. Conversely, three cases showed marked xanthoprotein elevations (125, 167, and 70, respectively), while the nonprotein nitrogen level was only 60, 64, and 37, respectively. In only two of the 36 cases, therefore, the nonprotein nitrogen determination was of greater significance in diagnosing uremia than was the xanthoprotein reaction, whereas in three cases the reverse was true.

All but two of these patients died in uremia. In one patient repeated tests indicated a decreasing xanthoprotein value, although the nonprotein nitrogen remained elevated, and he was subsequently discharged; the other patient had elevated xanthoprotein and nonprotein nitrogen values, but left the hospital before repeat studies could be performed. Of the 34 patients who died, two had xanthoprotein values below 50. In one patient in whom 12 xanthoprotein determinations were performed over a period of several months, the highest value was 50, although nonprotein nitrogen values ranged from 93 to 212 mg. per 100 c.e.; in the second patient the xanthoprotein value was 35, and the nonprotein nitrogen was 239.

Six patients with no clinical evidence of uremia showed xanthoprotein reactions over 60: one patient each with nephrosclerosis, nephrosis, rheumatic heart disease, lysol poisoning, diabetic gangrene, and chronic nephritis. All were discharged. The patient with lysol poisoning presented a xanthoprotein value of 325, and a nonprotein nitrogen value of 41. He was discharged before repeat studies were made. By way of contrast another patient with lysol poisoning was admitted, but his xanthoprotein reaction was only 42. The difference of these values in two similar conditions may, we believe, be explained by the differences in time at which blood was secured for this study. Thus, the first patient was hospitalized within several hours of attempted suicide, and blood was obtained shortly thereafter at a time when the blood serum contained considerable amounts of phenol bodies; whereas in the second patient blood was not obtained until several days after hospitalization, at a time when the patient was convalescing and his blood serum apparently was relatively free of phenol bodies.

*Cerebral Conditions.*—Twenty-two xanthoprotein determinations were performed on 21 patients with various cerebral conditions, including seven patients with hypertensive encephalopathy and 10 with cerebral vascular accidents. In the seven patients with hypertensive encephalopathy the nonprotein nitrogen was elevated, at times markedly so, but in none was the xanthoprotein reaction persistently or markedly increased. In only one patient did the xanthoprotein value reach 50, but on repeating the test it promptly fell to 20. In the 10 patients with cerebral vascular accidents the xanthoprotein reaction was low, despite the fact that the nonprotein nitrogen was elevated in all and markedly elevated in three.

*Nephritides.*—Excluding the patients with uremia, 30 xanthoprotein reactions were studied on 22 patients with acute and chronic renal pathology. In three patients the xanthoprotein value was elevated beyond 60, but these left the hospital before repeated studies could be done. In the remainder the xanthoprotein reaction was not significantly elevated, although nonprotein nitrogen

levels were very high in many instances. None of the nine patients with acute nephritis showed significantly increased xanthoprotein values, the highest being 31.

Nine xanthoprotein studies were carried out on seven patients with genitourinary conditions, excluding nephritis. In none was the xanthoprotein value significantly elevated, although in two patients with prostate pathology and urinary retention, the nonprotein nitrogen was markedly increased and impending uremia was suspected.

*Cardiac Conditions.*—Ninety-six xanthoprotein reactions were studied in 81 patients with cardiac disease complicated by cardiac failure. In only one patient was the reaction higher than 60. In two it was over 50, and in five it reached 50. The remainder were below 50. Six patients had xanthoprotein values of zero; in two of these patients the nonprotein nitrogen was markedly elevated, being 125 and 154, respectively.

*Gastrointestinal Conditions.*—Forty-six xanthoprotein reactions were performed on 43 patients with various gastrointestinal conditions, including 20 patients with carcinoma and marked emaciation and 13 with peptic ulcers, most of which demonstrated active bleeding. In no instance was the xanthoprotein reaction elevated beyond 50, the great majority of reactions being below 20. In 10 the nonprotein nitrogen was markedly elevated.

*Pulmonary Conditions.*—Fifty-two determinations were performed on 52 patients, mainly with pulmonary tuberculosis or pneumonia, and nothing significant was revealed. The highest value was 41, most values being 20 or lower. Many of these patients had moderately increased nonprotein nitrogen levels and several showed marked increases.

*Miscellaneous Conditions.*—Thirty-five xanthoprotein reactions were determined on 33 patients with various clinical conditions (alkalosis, alcoholism, arthritis, avitaminosis, pernicious anemia, etc.) not previously included. With exception of the patients with lysol poisoning and diabetic gangrene previously referred to, none showed significant xanthoprotein elevations, although in seven the nonprotein nitrogen was markedly increased.

Included are three patients with coma of undetermined origin. In none was the xanthoprotein or nonprotein nitrogen significantly increased. Five patients had diabetes mellitus, and nothing of significance could be determined from the xanthoprotein reactions.

#### SUMMARY AND CONCLUSIONS

1. Although the range of normal values of the xanthoprotein reaction is accepted as 15 to 35, our study indicates that a more correct normal range is 0 to 50. Xanthoprotein values over 60 should be considered abnormal in all conditions.

2. Our data indicate that the only specific condition with significantly increased xanthoprotein values is uremia, although lysol poisoning must be kept in mind as a possibility.

3. As an indicator of impending or true uremia, the xanthoprotein reaction seems to be as accurate as the level of nitrogenous retention in the blood. Thus, in our series the xanthoprotein reaction was an accurate indicator in 94.4 per

cent of cases, whereas the blood nonprotein nitrogen level was significantly elevated in 91.6 per cent. A low xanthoprotein reaction does not always rule out uremia (5.5 per cent of uremia cases) nor does a high value invariably mean uremia.

4. The xanthoprotein value does not in all instances prognosticate the course of the patient with uremia, although with one exception all those with uremia whose xanthoprotein reactions were 100 or over died. Conversely, low xanthoprotein values do not preclude a fatal termination of uremia.

5. With the exception of those cases of uremia in which both the xanthoprotein and blood nonprotein nitrogen were definitely elevated, there was no consistent correlation between the xanthoprotein reaction and the level of blood nitrogenous retention.

6. The xanthoprotein reaction is valuable in differentiating cerebral conditions simulating uremia (pseudo-uremia) from true uremia. This is particularly evident in studying patients with hypertensive encephalopathy in whom no instances of elevated xanthoprotein values were noted despite moderate elevation of nonprotein nitrogen in many instances.

7. A low xanthoprotein value is significant in cases of extrarenal azotemia as occurs in dehydration states, following hemorrhage into the gastrointestinal tract, etc.

8. Although the xanthoprotein reaction has some limitations, it is an excellent supplementary test in the study of uremia, suspect uremia, pseudo-uremia, coma, and nephritis. It consumes little time, is simple to perform, and is inexpensive.

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# THE EFFECT OF ANAPHYLACTIC SHOCK UPON BLOOD POTASSIUM AND CONCENTRATION\*

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CONFLICTING reports relative to the results obtained following employment of potassium salts in allergic conditions<sup>1-4</sup> and the apparent lack of an adequate rationale for such a procedure on the basis of experimental blood potassium findings<sup>5-7</sup> led us to further investigate the subject from an experimental standpoint.

The present experiments were designed to correlate the blood picture with the symptoms manifested in the animals rather than with the sensitizing dose, time intervals, or shock dose. This will be made clear in the presentation of our results, which show the effects of rapidly and slowly fatal anaphylactic shock on potassium, relative red blood cell volume, and plasma specific gravity.

## MATERIALS AND METHODS

Guinea pigs, weighing from 250 to 400 Gm., were used in this work. Blood obtained directly from the heart at various time intervals was drawn into a 2 c.c. tuberculin syringe, which contained a small amount of dry heparin (Connaught Laboratories, Toronto). Some of the heparinized blood was run immediately into a heavy wall 1 mm. capillary tube, 10 cm. long, which served as a hematocrit tube. Both the syringe and the tube were closed at both ends with broad rubber bands and centrifuged for fifteen minutes at 3,000 r.p.m. The hematocrit (relative red blood cell volume) was then read off directly in per cent by placing it on a millimeter scale.

The supernatant plasma in the syringe was used for both potassium and plasma specific gravity determinations. Potassium was determined in duplicate on 0.2 c.c. by the method of Truszkowski and Zwemer.<sup>8</sup> Plasma specific gravity was measured by the technique of Barbour and Hamilton,<sup>9</sup> and the proteins were calculated by the formula of Weech and co-workers.<sup>10</sup>

## EXPERIMENTAL

Except for two groups of the controls, all animals were sensitized with 0.25 c.c. of horse serum. A second dose of horse serum was administered one to four weeks later by the intraperitoneal route. The "shock" animals fell into three clinical groups: (a) those that showed no evidence or only questionable evidence of shock (Table I); (b) those that died of anaphylactic shock after a period of

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TABLE I

ANIMALS RECEIVING TWO DOSES OF HORSE SERUM BUT SHOWING LITTLE OR NO CLINICAL EVIDENCE OF SHOCK

(Average time interval between initial and final values is one hour.)

| GUINEA PIG NO.                                  | 1         | 2        | 5        | 7        | 32       |
|---|-----------|----------|----------|----------|----------|
| Body weight                                     | 254       | 204      | 266      | 317      | 307      |
| Shock dose of horse serum per 100 Gm. of weight | 0.78 c.c. | 0.9 c.c. | 0.9 c.c. | 0.6 c.c. | 0.1 c.c. |
| Sensitization interval                          | 1 wk.     | 1 wk.    | 2 wk.    | 2 wk.    | 3 wk.    |
| <i>Potassium (mg. per 100 c.c.)</i>             |           |          |          |          |          |
| <i>Values</i>                                   |           |          |          |          |          |
| Initial   | 35.4      | 25.6     | 27.0     | 28.2     | 28.0     |
| Intermediate                                    | 28.5      | 32.8     | 26.7     | 33.8     | 29.2     |
| Final   | 26.5      | 28.8     | 28.8     | 32.8     | 26.7     |
| <i>Hematocrit (per cent)</i>                    |           |          |          |          |          |
| Initial   | 37.1      | 36.0     | 46.4     | 41.5     | 44.3     |
| Intermediate                                    | 31.1      | 24.0     | 38.5     | 37.3     | 38.0     |
| Final   | 37.0      | 26.4     | 31.1     | 33.3     | 35.5     |
| <i>Protein (Gm. per 100 c.c.)</i>               |           |          |          |          |          |
| Initial   | 4.39      | 5.41     | 5.03     | 4.39     | —        |
| Intermediate                                    | 3.88      | 4.93     | 4.05     | 4.32     | —        |
| Final   | 4.52      | 4.73     | 3.37     | 3.74     | —        |

TABLE II

ANIMALS RECEIVING TWO DOSES OF HORSE SERUM AND SHOWING SHOCK OF SLOW ONSET AND MODERATE DEGREE

(Average time interval between initial and final values is four hours. Complete findings on animal No. 10 are shown in Graph 4.)

| GUINEA PIG NO.                                  | 9        | 10       | 13       | 39       | 40       | 41       |
|---|----------|----------|----------|----------|----------|----------|
| Body weight                                     | 305      | 285      | 319      | 270      | 344      | 431      |
| Shock dose of horse serum per 100 Gm. of weight | 0.8 c.c. | 0.8 c.c. | 1.2 c.c. | 1.5 c.c. | 1.5 c.c. | 1.5 c.c. |
| Sensitization interval                          | 2 wk.    | 2 wk.    | 3 wk.    | 3 wk.    | 3 wk.    | 3 wk.    |
| <i>Potassium (mg. per 100 c.c.)</i>             |          |          |          |          |          |          |
| <i>Values</i>                                   |          |          |          |          |          |          |
| Initial   | 27.9     | 26.4     | 25.7     | 30.7     | 33.1     | 22.2     |
| Intermediate                                    | 29.9     | 29.9     | 29.2     | 37.0     | 29.5     | 18.8     |
| Final   | 39.6     | 46.5     | 43.0     | 38.8     | 37.0     | 27.1     |
| <i>Hematocrit (per cent)</i>                    |          |          |          |          |          |          |
| Initial   | 46.0     | 45.2     | 43.7     | 35.0     | 45.8     | 44.1     |
| Intermediate                                    | 50.0     | 25.9     | 50.0     | 25.9     | 34.7     | 42.9     |
| Final   | 38.9     | 38.8     | 46.7     | 30.5     | 32.3     | 49.0     |
| <i>Protein (Gm. per 100 c.c.)</i>               |          |          |          |          |          |          |
| Initial   | 4.49     | 5.51     | 5.06     | 4.56     | 5.85     | 5.85     |
| Intermediate                                    | 3.57     | 4.59     | 4.22     | 4.42     | 4.93     | 5.12     |
| Final   | 3.78     | 4.42     | 4.04     | 4.69     | 5.17     | 5.27     |

TABLE III

ANIMALS RECEIVING TWO DOSES OF HORSE SERUM AND SHOWING SHOCK OF RAPID ONSET AND SEVERE DEGREE\*

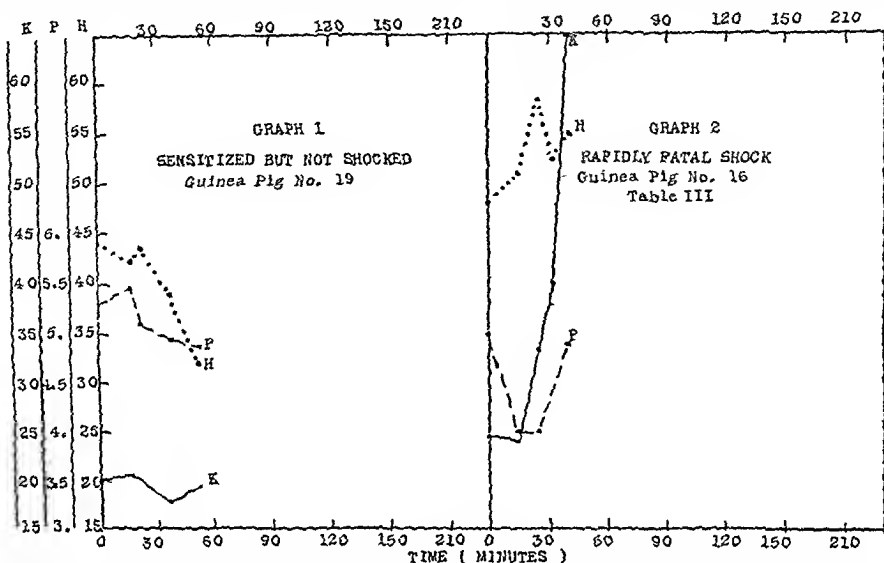
(Average time interval between initial and final values is forty-five minutes. Complete findings of animal No. 16 are shown in Graph 2.)

| GUINEA PIG NO.                                  |  | 6         | 8        | 14       | 16       | 17       | 25       | 28       | 27       | 29       | 33       |
|---|--|-----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Body weight                                     |  | 267       | 222      | 300      | 368      | 231      | 304      | 276      | 283      | 349      | 320      |
| Shock dose of horse serum per 100 Gm. of weight |  | 0.75 c.c. | 0.9 c.c. | 2.2 c.c. | 2.7 c.c. | 3.1 c.c. | 1.5 c.c. | 1.5 c.c. | 1.5 c.c. | 2.0 c.c. | 2.0 c.c. |
| Sensitization interval                          |  | 2 wk.     | 2 wk.    | 3 wk.    | 4 wk.    | 4 wk.    | 2 wk.    | 3 wk.    | 2 wk.    | 3 wk.    | 4 wk.    |
| <i>Potassium (mg. per 100 c.c.)</i>             |  |           |          |          |          |          |          |          |          |          |          |
| Initial   |  | 31.7      | 28.0     | 30.4     | 24.5     | 24.1     | 31.3     | 27.0     | 31.5     | 31.3     | 30.3     |
| Intermediate                                    |  | Insuf.    | Insuf.   | 37.5     | 33.5     | 29.7     | 31.4     | 31.0     | 34.2     | 35.4     | 34.5     |
| Final   |  | Insuf.    | 42.8     | 55.6     | 65.8     | 52.0     | 37.0     | 40.0     | Insuf.   | 41.3     | 34.8     |
| <i>Hematocrit (per cent)</i>                    |  |           |          |          |          |          |          |          |          |          |          |
| Initial   |  | 38.4      | 40.5     | 44.0     | 48.1     | 49.1     | 47.6     | 43.6     | 44.6     | 43.7     | 49.6     |
| Intermediate                                    |  | Insuf.    | 28.5     | 44.7     | 58.6     | 46.7     | 41.6     | 42.1     | 38.9     | 47.5     | 62.7     |
| Final   |  | 44.4      | 42.8     | 57.6     | 55.0     | 53.4     | 36.5     | 42.2     | 48.2     | 48.2     | 61.2     |
| <i>Protein (Gm. per 100 c.c.)</i>               |  |           |          |          |          |          |          |          |          |          |          |
| Initial   |  | 4.46      | 4.93     | 4.42     | 5.27     | 5.14     | 4.90     | 4.63     | 3.88     | 4.69     | 4.97     |
| Intermediate                                    |  | Insuf.    | 4.63     | 4.97     | 4.02     | 5.14     | 4.42     | 4.49     | 4.42     | 4.22     | 4.52     |
| Final   |  | 4.93      | 5.92     | 4.90     | 4.97     | 5.48     | 3.98     | 4.39     | Insuf.   | 5.03     | 4.49     |

\*Death occurred within one hour.



three or more hours (Table II, Graph 4); and (c) those that went into shock and were dead within an hour (Table III, Graph 2). The control series were of four types: (a) three animals sensitized but not shocked, and bled rapidly at intervals corresponding to those undergoing intense shock, one of which is shown

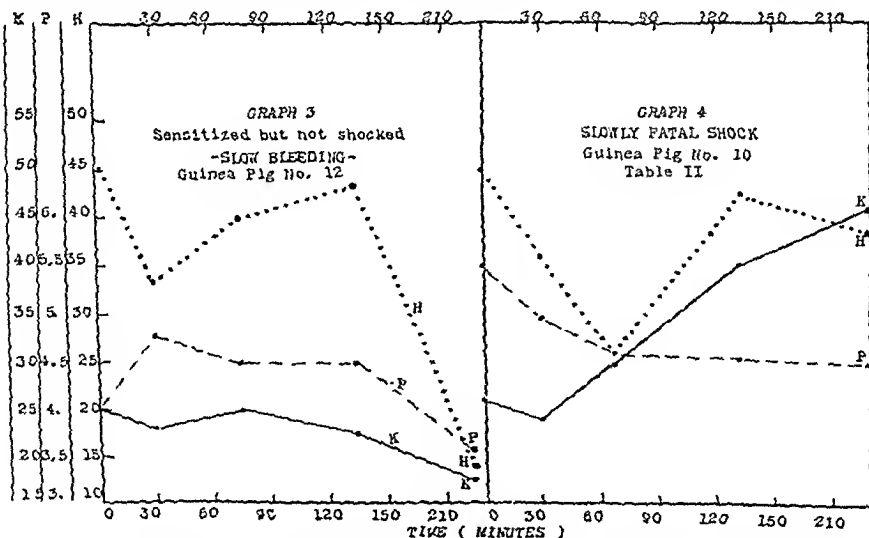


Graphs 1 and 2.—Note that potassium rise in shocked animal is out of proportion to hemo-concentration.

H = hematocrit (packed red blood cell volume per cent).

P = plasma proteins (grams per 100 c.c. as calculated from plasma specific gravity).

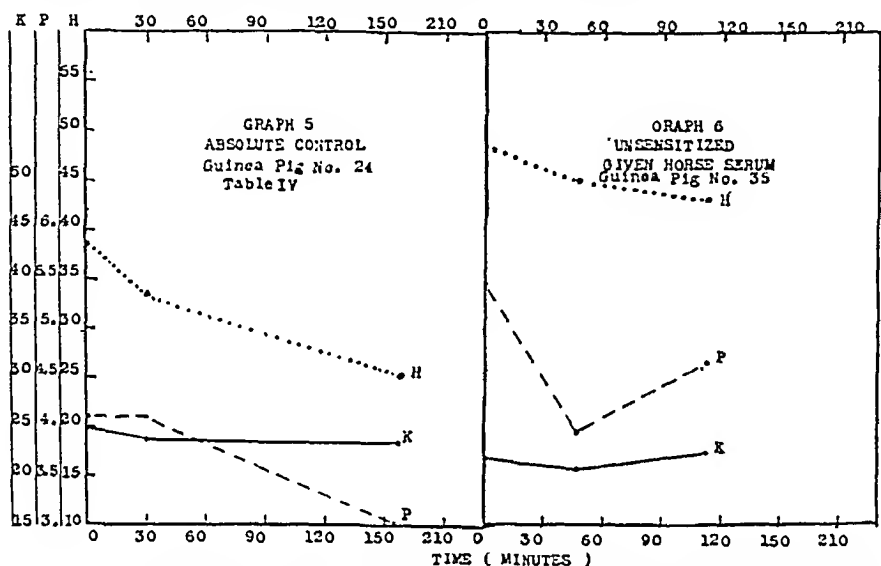
K = potassium (milligrams per 100 c.c. of plasma).



Graphs 3 and 4.—Note the steady rise in plasma potassium despite some hemodilution.

in Graph 1; (b) three bled at less frequent intervals which corresponded to slow shock (example in Graph 3); (c) three guinea pigs given large amounts of horse serum without previous sensitization (example in Graph 6); and (d) three controls which had received no injections of any kind and were used to check the effects of blood loss and accuracy of technique (Table IV, Graph 5).

Animals in which anaphylactic shock of rapid onset and marked intensity was produced had a pronounced elevation in plasma potassium which was greater than could be accounted for by the hemoconcentration as measured by red blood cell volume and plasma protein. This was in contrast to the control group which with equally rapid sampling showed hemodilution, lowered plasma protein and, in some cases, a lowered potassium. The changes were roughly proportional to the amount of blood lost by sampling; this blood loss is an important factor in increasing the severity of anaphylaxis in small animals.<sup>11</sup>



Graphs 5 and 6.—Note the constancy of plasma potassium despite hemodilution resulting from bleeding.

Examples from the significant groups are given in Graphs 1 to 6, and demonstrate quite clearly the effect of anaphylactic shock on the three blood constituents studied. If one compares Graphs 1 with 2, the difference between the shocked and unshocked animals, in so far as plasma potassium is concerned, is quite striking. The same may be said regarding Graphs 3 and 4, the sensitized unshocked animal showing essentially only those changes which may be attributed to blood loss, whereas the shocked animal shows a steadily rising plasma potassium level.

Also, if one compares all the graphs of animals in the control groups (Graphs 1, 3, 5, 6), the general similarity is apparent.

#### DISCUSSION

A rise in plasma potassium has been shown to be a feature of anaphylactic shock,<sup>5-7, 12</sup> of neurogenic and traumatic shock,<sup>13, 14</sup> and of acute allergic reactions.<sup>15</sup> Our results confirm the plasma potassium rise during the course of anaphylactic shock and show it to be definitely out of proportion to the degree of hemoconcentration and increased plasma density.

The findings in regard to changes in blood concentration are confirmatory of the work of Petersen and Levinson,<sup>16</sup> of Simonds,<sup>17</sup> and of Manwaring and

others,<sup>18-21</sup> most of whom ascribe these changes to disturbances of endothelial permeability occurring during the course of the anaphylactic reaction.

Black and Kemp<sup>22</sup> reported a consistent increase in plasma density during the course of anaphylactic shock in guinea pigs. Our findings do not agree, but the time factor, or the amount of blood taken, may explain this apparent contradiction. Other authors report a lack of constant variation in total protein during the course of anaphylactic shock, but note consistent shifts in the albumin-globulin ratio and in fibrin values.<sup>23-25</sup>

TABLE IV

## ANIMALS RECEIVING NO HORSE SERUM

(Average time interval between initial and final values is two hours. Complete findings of animal No. 24 are shown in Graph 6.)

| GUINEA PIG NO.                                  | 21   | 24   | 37   |
|---|------|------|------|
| Body weight                                     | 269  | 233  | 425  |
| Shock dose of horse serum per 100 Gm. of weight | 0    | 0    | 0    |
| Sensitization interval                          | 0    | 0    | 0    |
| <i>Potassium (mg. per 100 c.c.)</i>             |      |      |      |
| <i>Values</i>                                   |      |      |      |
| Initial   | 27.3 | 24.7 | 23.7 |
| Intermediate                                    | 27.0 | 23.7 | 25.3 |
| Final   | 28.4 | 23.1 | 26.6 |
| <i>Hematocrit (per cent)</i>                    |      |      |      |
| Initial   | 42.8 | 38.9 | 46.0 |
| Intermediate                                    | 40.4 | 33.3 | 42.6 |
| Final   | 38.3 | 25.4 | 40.2 |
| <i>Protein (Gm. per 100 c.c.)</i>               |      |      |      |
| Initial   | 4.83 | 4.15 | 5.27 |
| Intermediate                                    | 3.91 | 3.96 | 5.24 |
| Final   | 3.78 | 2.96 | 4.69 |

The mechanism of the potassium rise still remains to be demonstrated. Since potassium is the principal intracellular base, as contrasted to sodium, which is the essential basic ion of plasma and interstitial fluid, it seems reasonable to assume that an increase of plasma potassium represents a loss of potassium by cells due to either a change in cell membrane permeability or a disruption in the hypothetical intracellular potassium colloid complex, or both.

Since it has been shown that injections of potassium salts are capable of producing many symptoms of shock,<sup>6, 27</sup> it seems probable that the increase in plasma potassium, occurring during the course of anaphylactic shock, accounts in some measure for the symptoms which are characteristic of this condition. In view of the close relationship between anaphylaxis and allergy, these findings suggest that the use of large amounts of potassium in allergic conditions is contraindicated rather than indicated.

## SUMMARY

1. Thirty-four guinea pigs were used to determine the effect of anaphylactic shock upon plasma potassium and blood concentration in terms of plasma protein and red blood cell volume per cent.

2. A constant rise in plasma potassium was demonstrated, together with a fairly constant hemoconcentration in terms of red blood cell volume per cent. The potassium rise was relatively much greater than the degree of hemoconcentration and was roughly proportional in degree to the severity of shock. No constant change in plasma protein was demonstrable.

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## THE CHEMISTRY OF INFECTIOUS DISEASES\*

### III. POLAROGRAPHIC STUDIES OF THE BEHAVIOR OF NORMAL AND PNEUMOCOCCUS INFECTED DOG SERA TOWARD DENATURATION AGENTS AND ENZYMES

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VERY little information is available on the low molecular weight protein derivatives of serum. Lack of convenient methods of handling proteoses, peptones, and polypeptides is responsible for their neglect in studies of protein metabolism.

The lower derivatives (peptones and polypeptides) are not precipitated by salting-out, and are soluble in trichloroacetic and sulfosalicylic acids, but the addition of tungstic or phosphotungstic acid to a sulfosalicylic acid filtrate of serum will give a voluminous precipitate.

It is not known whether the serum protein derivatives of low molecular weight possess any physiologic activity of their own. Recent evidence indicates, however, that the peptone fraction† of blood serum may play a part in health and disease. It has been demonstrated recently<sup>1</sup> that during experimental type I pneumonia in dogs this peptone fraction greatly increases and remains abnormally high long after clinical evidences of infection have disappeared (fever, complete resolution of the congested areas of the lung as shown by x-ray, and the physical well-being of the animal). A positive polarographic cancer reaction according to Brdicka's technique<sup>2</sup> probably also depends upon the relative amounts of serum peptones.

The abnormal increase in the peptone fraction of the blood during pneumococcal infection may be only a reflection of a general increased metabolism, possibly associated with fever, or it may be concerned with the catabolism of the proteins at the site of infection. These two possibilities as to the probable source of the increased peptone fraction during infections are under investigation. However, there is another possible explanation. If one or more of the serum proteins should lose their normal stability under the stress imposed upon the organism by the invading microorganism, an abnormal tendency to disintegrate may occur. Certain facts suggest that this might actually take place, and this paper deals with the results of the investigation.

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†Peptone fraction will be defined as that part of blood serum which is responsible for the typical polarographic protein wave in 0.5% NaCl-buffer after precipitation of the proteins by the sulfosalicylic acid.

## EXPERIMENTAL

The stability of dog sera toward denaturation and enzymatic hydrolysis before and after experimental type I pneumococcus infection was determined polarographically by comparing the relative rates at which the polarographic wave heights of normal and of infected sera changed during denaturation. Preliminary experiments with urea, guanidine hydrochloride, alkali, and wetting agents showed that 8 M concentrations of the first two denaturing agents inhibited the formation of the typical polarographic wave to such an extent as to preclude their use in this study, while 1 N potassium hydroxide and Aerosol OT gave satisfactory results with the polarograph.

The dogs used in this study were normal, adult mongrels, weighing 34 to 48 pounds, which had lived in our kennel for at least five weeks prior to infection. Diet, care, the method of blood sampling, and the mode of infection with type I pneumococci have been described previously.<sup>1</sup> Denaturation of the sera was obtained by the addition of 10 c.c. of 1 N potassium hydroxide (or 10 c.c. 0.025 M Aerosol OT) to 4.0 c.c. of serum at room temperature. After various intervals, 1.4 c.c. of the mixture were added to 1 c.c. of 20 per cent sulfosalicylic acid and filtered after standing for five minutes. One-half cubic centimeter of the filtrate was electrolyzed in 5 c.c. of the previously described cobaltic ammonium chloride buffer<sup>1</sup> at 1/100 sensitivity of the galvanometer. The zero time of action was obtained by adding 2 c.c. of a 1:1 mixture of 1 N potassium hydroxide and 20 per cent sulfosalicylic acid directly to 0.4 c.c. of serum, filtering after five minutes' standing, and treating 0.5 c.c. of the filtrate as described above. The conditions of the enzymatic hydrolysis experiments were so chosen that just enough enzyme was present to insure a polarographically measurable digestion under optimum conditions of pH and temperature without having the waves produced by the enzymes themselves mask those produced by the partially digested proteins. The following procedure was finally adopted: 2 c.c. of 10 mg. per cent pepsin (Difco, spongy granular 1:3,000) in 1 N hydrochloric acid were added to 10 c.c. of either normal or infected serum. After a definite period of incubation at 39° to 40° C., 0.48 c.c. of the solution was withdrawn, added to 2 c.c. of 1:1 mixture of 1 N potassium hydroxide and 20 per cent sulfosalicylic acid. After standing for five minutes the solution was filtered. One-half cubic centimeter of the filtrate was analyzed polarographically in 5 c.c. of the cobaltic ammonium chloride buffer with the galvanometer sensitivity set at 1/100. For the tryptic digestion, 8 c.c. of serum were added to 2 c.c. of a 1 per cent trypsin (Difco, 1:110) solution in M/15 phosphate buffer at pH 8. Otherwise the procedure outlined for the peptic digestion was followed, except that 0.5 c.c. of the digestion mixture was added to 2 c.c. of the potassium-sulfosalicylate instead of 0.48 c.c.

All polarograms are self-recorded photographs of the galvanometer deflection. The increase in galvanometer deflection between -0.9 volts and the minimum of the double wave (-1.65 volts) determines the wave height. Any change from the normal is interpreted as being due to concentration changes in polarographic active groups of the peptone fraction of the serum after infection.

The rate at which the wave height increased, above that given by normal serum, by such criteria, would be indicative of an increased rate of denaturation of the serum proteins after infection.

### RESULTS

*Alkaline Denaturation.*—Two typical sets of polarographic recordings of the peptone fractions from alkali-treated dog sera are reproduced in Fig. 1. Curve I, Fig. 1, represents the polarograms obtained from the filtrates of a normal dog serum, while curve II corresponds to that of the serum from the same dog after a mild attack of experimental pneumococcal pneumonia. The severity of infection in all cases is judged by fever, lung congestion as shown by x-ray, and by the general appearance of the animal. Upon the addition of

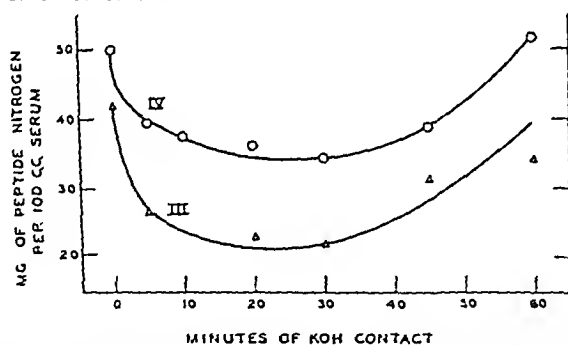
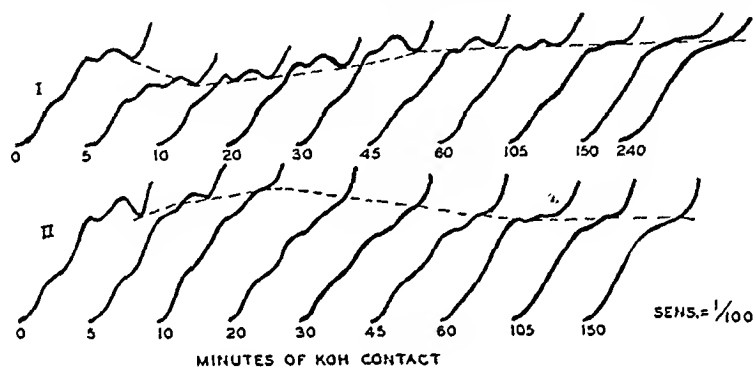


Fig. 1.—Polarograms and nitrogen values of the serum peptone fraction of alkalidenatured dog sera before (I and III) and after (II and IV) type I pneumococcus infection.

alkali to normal serum, a decrease in wave height occurs in the sulfosalicylic acid-soluble serum fraction during the first five to ten minutes of alkali contact, followed by a rise until a fairly constant wave height is reached at sixty minutes (curve I). Further contact with alkali tends to destroy the double wave, a single wave appearing instead after the 150th to 240th minute. Denaturation of the serum from the same dog after infection (curve II) follows an entirely different course. A rise in wave height occurs during the first five to ten minutes of alkali contact, followed thereafter by a fall to near the original level of the untreated serum (zero time) which, incidentally, is also approximately that obtained from normal serum after sixty minutes' denaturation. The two polarographic denaturation curves are thus entirely different from each other.

Wherever the peptone fraction of normal serum shows a rise in wave height, the corresponding one from the infected serum decreases, and vice versa. There exists still a further difference in the polarograms of the two types of sera. The change from a double wave to a single wave occurs much sooner when the serum from an infected dog is denatured. Depending upon the severity of infection, this change in the structure of the polarograms has appeared as early as forty-five minutes after alkali addition, and always after sixty to one hundred and five minutes, while normal serum never showed the disappearance of the double wave prior to one hundred and fifty minutes of alkali contact.

If the assumption is correct that an alteration in the structure of the serum proteins occurs during infection and is responsible for the observed differences in wave heights during the early, and again in the late periods of alkali contact, then it should follow that the sudden rise in wave height during the first five to ten minutes after potassium hydroxide addition should be most pronounced in cases of severe infections, and less so during the early periods of pneumococcal invasion, when lung inflammation is still restricted. To test this, the spreading of the infection in the lungs was followed by x-ray, and blood samples were drawn at various stages of infection, the serum was denatured as described, and the wave heights of the peptone-polarograms were plotted against time of alkali content. In this manner a series of denaturation curves are obtained (Fig. 2) which fully substantiate the original assumption. The more severe the infection, the greater and more abrupt is the initial rise in wave height, while the polarograms of the normal serum are again entirely opposite. The zero time values in Fig. 2 express, in terms of polarographic wave heights, the pre-existing concentrations of peptone material of the sera prior to denaturation and confirm our previous observations<sup>1</sup> that an increase in the serum-peptone fraction takes place during pneumonia. Interestingly enough, the blood sample drawn twenty-four hours after infection still exhibits a decrease in wave height during the first ten minutes after alkali addition, although it is not as pronounced as for normal serum, while its zero time value demonstrates that an accumulation of peptone material already has begun.

In polarography, changes in wave height merely indicate that concentration changes have taken place in the material which are responsible for the wave. Under our experimental conditions only cystine-containing proteins will give the wave. If the peptones which accumulate during pneumonia, and which are split off from the serum proteins by alkaline denaturation always possess the same average cystine composition, then the nitrogen content of the peptone fraction should fluctuate in a similar manner to that of the corresponding polarograms. Micro-Kjeldahl nitrogens were, therefore, run on some of the peptone fractions at various time intervals of alkaline denaturation, the peptone nitrogen being determined as the difference between the nitrogen value after sulfosalicylic acid and after tungstic acid precipitation of the serum. Curves III and IV in Fig. 1 are graphs of such analyses performed on the serum filtrates which correspond to the polarographic curves I and II of Fig. 1, respectively. It is evident from an inspection of curves II and IV that the peptone fraction of the infected serum (curve IV), as expected, contains more peptone nitrogen



throughout the entire denaturation range than the normal one. Entirely unexpected were the findings that the nitrogen of both the normal and the infected serum filtrates decreased during the first twenty minutes of alkali contact, and then increased thereafter until, after sixty minutes, approximately the initial values were obtained. The changes in peptone nitrogen of normal denatured serum thus ran roughly parallel to those in polarographic wave height. However, this relationship does not hold for those from infected serum. The possible significance of this discrepancy will be discussed later, but it should be pointed out that a direct dependency of wave height to peptone concentration can be readily demonstrated. If, for instance, the observed lower wave height of normal, undenatured serum (i.e., zero times in Figs. 1 and 2) is due to less peptone material than is found in an equal volume of untreated serum of the

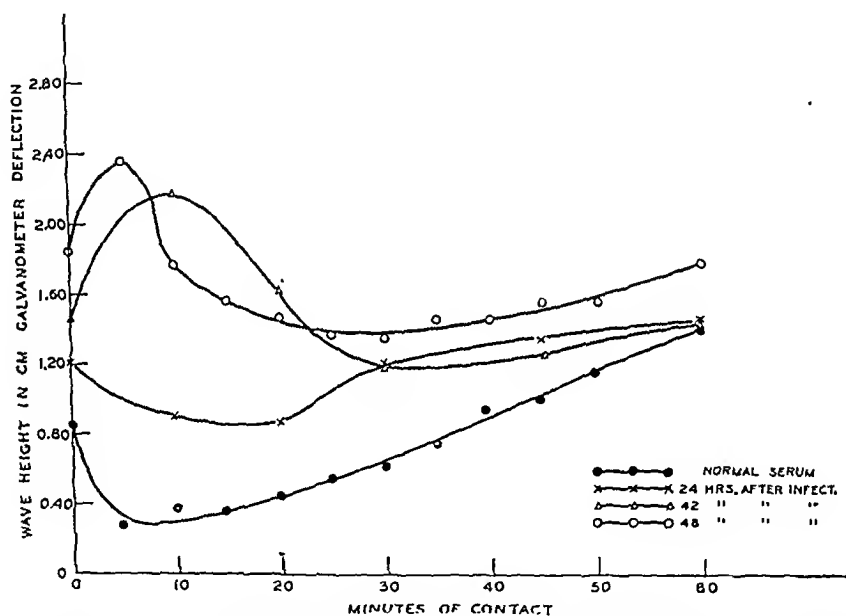


Fig. 2.—The influence of pneumococcus infection upon the characteristics of normal serum-peptone curves during alkaline denaturation.

same dog after infection, then it should be possible to match the waves obtained from the latter by merely increasing the amounts of the former. The results of such experiments are shown in Fig. 3. Each polarogram in the upper row was obtained by electrolyzing 0.5 c.c. of serum filtrate (equivalent to 0.083 c.c. of original serum), the blood of which was drawn at various stages of infection. The polarograms of the lower row were obtained from normal serum only, except that the serum concentrations were increased above 0.083 c.c. in such manner that each wave matched in height one of the current-voltage curves of the infected sera. It is evident that the polarograms due to 0.100 c.c., 0.133 c.c., 0.166 c.c., and 0.199 c.c. of normal serum are identical, within the error of the method, with those from 0.083 c.c. of sera drawn from the same dog, twenty-four, forty-two, forty-eight, and ninety hours after infection. Similarly, normal and infected peptone polarograms of denatured sera can be matched, either by increasing the concentration of the former or by decreasing that of the latter.

When different denatured sera are compared, the length of time during which the alkali acts must be the same, since alkali contact eventually causes all peptone polarograms, whether obtained from normal or from infected sera, to lose their double wave. The sera from which the curves in Fig. 4 were obtained were consequently denatured in each case for forty-five minutes with 1 N potassium hydroxide. The infected serum was drawn from the same animal during the

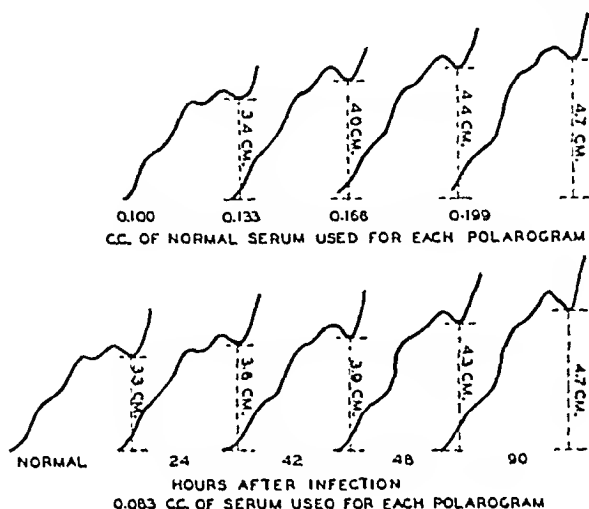


Fig. 3.—The effect of concentration of undenatured serum filtrates from normal and infected dogs upon the polarographic wave height.

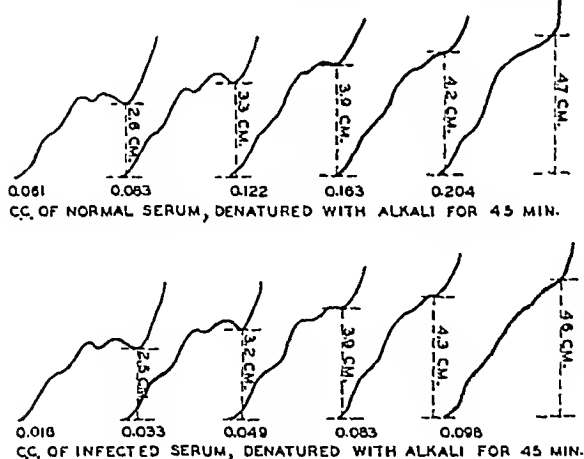


Fig. 4.—The effect of concentration of denatured serum filtrates from normal and infected dogs upon the polarographic wave height and wave structure.

course of an infection of medium severity. In order to match the polarograms from 0.061 e.e., 0.083 e.e., 0.122 e.e., 0.163 e.e., and 0.204 e.e. of normal, denatured serum, filtrates containing 0.016 e.e., 0.033 e.e., 0.049 e.e., 0.083 e.e., and 0.098 e.e. of infected serum, respectively, were required. The double wave begins to straighten out when the peptone fraction from 0.163 e.e. of normal or

0.083 c.c. of abnormal serum is electrolyzed, and it disappears completely, in favor of a single wave, when the concentrations of normal and infected serum reach 0.204 c.c. and 0.098 c.c., respectively. These experiments (Figs. 3 and 4) clearly show: (1) that wave height differences in the polarograms of the peptone fraction from dog sera, before and after pneumococcal infection, reflect concentration changes in the polarographically active principle, both for untreated as well as for denatured sera; (2) that, within the limits of concentrations studied here, no alteration in the structure of the polarographic double wave occurs with increasing concentrations of peptone material from untreated serum, but that alkali-denatured serum reaches a limit in concentration above which the double wave changes over to a single wave; (3) that the disappearance of the double wave occurs at considerably lower serum concentrations of infected serum than of normal serum.

*Wetting Agents.*—Alkaline denaturation at room temperature unquestionably produces more than one change in the structure of a protein molecule. Rupture of secondary linkages, partial hydrolysis of peptide bonds, and a breakdown of disulfides undoubtedly occur. Denaturation by wetting agents, such as Aerosol OT, Duponol PC, Duponol 80, and others<sup>3</sup> probably proceeds to a much more limited extent, possibly involving only the liberation of sulfhydryl groups from cystine. Since the polarographic protein wave is supposedly given by -SH-protein as well as by -S-S-protein groups, a preliminary change of the oxidized form to the reduced form by wetting agents should not increase the polarographic wave height, unless a liberation of previously unavailable thiol groups had occurred. If cyclic sulfur compounds of the thiazoline type are part of a protein molecule,<sup>4</sup> a liberation of new thiol groups by the action of alkali is conceivable and would at least partially account for the increase in polarographic active groups. No information as to the action of wetting agents on these heterocycles is available. It is, however, unlikely that the wetting agents would liberate thiol groups from thiazolines. When the influence of Aerosol OT on serum was studied, it was found that the polarographic peptone wave, even after twenty-four hours of Aerosol OT contact, was identical with that of the same serum at zero time of Aerosol OT action. This was true for normal serum as well as for the serum of the same dog after a severe pneumococcal infection, although the relative heights of the two sera naturally differed, that from normal serum being the lower.

*Enzymes.*—The observed differences in the behavior of normal and of infected sera toward alkali, and the consequent belief that an alteration in the chemical structure of the proteins had occurred during infection, prompted us to investigate their enzymatic hydrolysis. It was hoped that pepsin and trypsin would digest normal serum proteins at a rate measurably different from that of infected serum; however, no differences were observed. Consequently, only the digestion polarograms of normal serum are shown in Fig. 5, where curve I represents the peptic digestion and curve II the tryptic one. The first current-voltage curve in each series is that of the blank, obtained by substituting saline for the serum. Its wave height must be subtracted from the serum-digestion polarograms if quantitative comparisons are to be made. Qualitatively, it is

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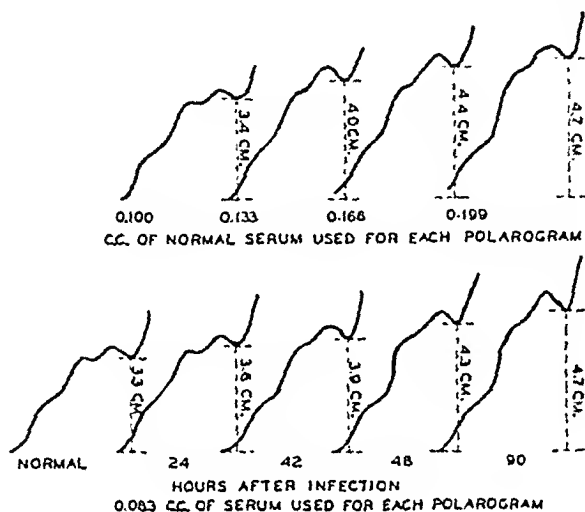


Fig. 3.—The effect of concentration of undenatured serum filtrates from normal and infected dogs upon the polarographic wave height.

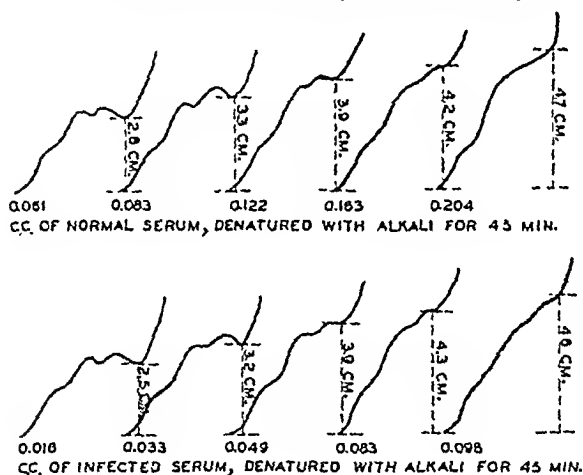


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quite evident that the polarographic technique lends itself well to proteolytic studies. Any increase in wave height above that of the zero time is due to proteolysis and is, therefore, a measure of the degree of enzymatic degradation that occurs. Furthermore, it permits an approximate estimation of those protein degradation products which were formed during digestion. These are not precipitable by sulfosalicylic acid but have a larger molecular weight than that of free cystine and cysteine, since these two amino acids in the free state give only a wave in cobaltous ammonium chloride buffer but not in the cobaltic buffer used here. Special attention should be called to the straightening out

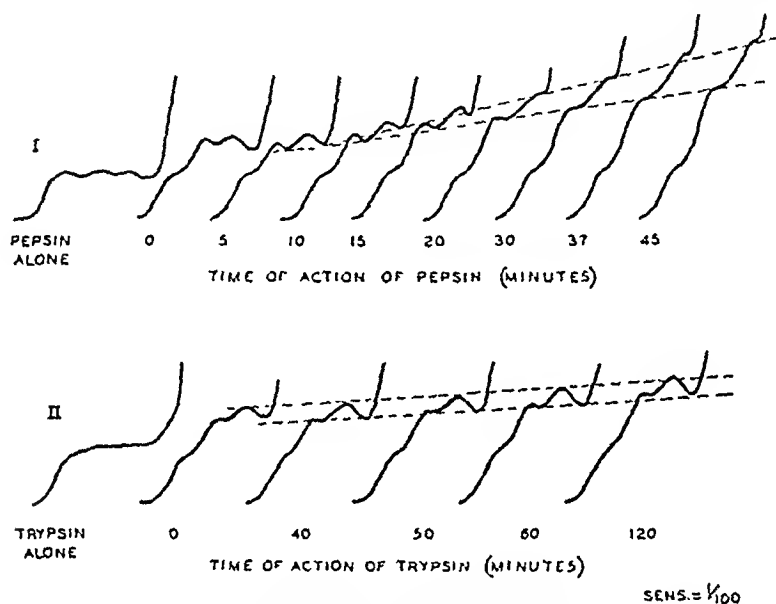


Fig. 5.—Polarograms of deproteinized dog sera at various stages of peptic (I) and tryptic (II) digestion at 10° C.

of the double wave during peptic digestion of serum, and of the failure to do so in case of tryptic digestion. A change in the structure of the polarographic protein double wave has so far been associated with denaturation and not with hydrolysis. Peptic activity seems to resemble denaturation in this respect, because the straightening out effect is also obtained by heat denaturation<sup>5</sup> or ultraviolet light irradiation<sup>6</sup> of serum albumin. Interestingly enough, ultraviolet irradiation of egg albumen causes a decrease in wave height<sup>6</sup> instead of the increase observed with serum albumin.

The relatively slower digestion of the serum proteins by trypsin than by pepsin is probably due to differences in the enzyme preparations. Since we were interested only in the relative rates of digestion of normal and of infected serum by the same enzyme preparation, no attempt was made to compare the different enzymes.

#### DISCUSSION

It is well known that when alkali is added to native proteins, denaturation occurs. This is followed by cleavage of the denatured protein into fragments, the sizes of which decrease as the action of the alkali proceeds. These degrada-

tion products are not very clearly defined, but have been classified as metaproteins, proteoses, peptones, polypeptides, and peptides, according to certain arbitrarily defined characteristics. Those products of a molecular weight smaller than that of metaproteins are not precipitated by sulfosalicylic acid; consequently the magnitudes of the polarographic wave heights, as shown in Figs. 1 and 2, are a measure of the smaller protein molecules only. With these facts in mind an attempt will be made to present a mechanism which will describe the course of the alkaline degradation of normal and of pneumococcus-infected dog sera.

If alkali were added to a serum which contained native proteins only and which was free of all other protein degradation products, the sulfosalicylic acid filtrates of such a serum would give no polarographic waves until the first molecules of proteoses were formed. There is an induction period, the length of which would be determined by the rate at which native proteins become hydrolyzed to the metaprotein state ( $k_1$ ) and by the average of the rates of degradation of the latter to proteoses ( $k_2$ ). On the other hand, if the higher proteins coexisted with proteoses and peptones in untreated serum, then the filtrate after the addition of sulfosalicylic acid would give a polarographic wave, the magnitude of which would reflect the concentration of pre-existing peptone molecules. The course of the reaction after the addition of alkali to such a serum would be determined by the relative amounts of pre-existing native proteins, metaproteins, and peptones, as well as by the relative rates of  $k_1$ ,  $k_2$ , and  $k_3$ , the latter being the average of the rates of the destruction of the sulfosalicylic acid-soluble protein fractions to particles sufficiently small not to give a polarographic wave in trivalent cobalt buffer. We know that cystine has no influence on the wave in this medium and that glutathione gives a barely visible wave. Preliminary work, which we hope to report soon in detail, has shown that if definite values are assigned to the rate constants in such a manner that  $k_1 > k_2 > k_3$ , then graphs can be plotted which show the same characteristics in their shapes as the curves given in Figs. 1 and 2, both for normal and for infected sera. In the case of normal serum, which contains relatively small concentrations of peptones, a pronounced induction period would be expected during which no new proteoses enter the sulfosalicylic acid filtrates. However, during this induction period, some of the pre-existing peptones of very low molecular weight are changed to such an extent that they become polarographically inactive, causing a gradual lowering of the wave heights of the polarograms. This period is followed by an interval of peptone accumulation, since the rate of proteose formation from proteins has been postulated as being more rapid than the destruction of peptones, and it is manifested by a rise in the wave height. After all the proteins have been degraded, the polarograms begin to decrease. Experiments carried out, but not included in Figs. 1 and 2, wherein the alkali was allowed to act on the serum proteins for a period of twenty-four hours, have shown that filtrates obtained after sulfosalicylic acid precipitation no longer produce the characteristic polarograms. Further evidence of the correctness of such a reaction mechanism is also borne out by our observation that the nitrogen values of the filtrates decrease during the first fifteen minutes of alkali contact, but then rise again to the original value or above it (Fig. 1, curve III).

The large accumulation of peptone material in the serum of infected dogs prior to the addition of alkali suggests that a degradation of native serum proteins had occurred *in vivo*. Under these circumstances it is reasonable to assume that the sulfosalicylic acid-insoluble fraction does not consist entirely of native proteins, but is a composite of native, denatured, and metaproteins. In such a case no induction period should be expected, as the experimental evidence confirms, and the formation of new proteoses would exceed the rate of peptone destruction, since it was postulated that  $k_1 > k_2 > k_3$ . Such a mechanism, therefore, accounts for the initial high level in polarographic wave height at zero time, the rapid initial rise after the addition of alkali, and a more sharply defined fall after all of the macroproteins have been converted to sulfosalicylic acid-soluble molecules. The initial fall in the nitrogen values of the peptone fraction of the infected serum, as shown in Fig. 1, curve IV, is the only datum not in complete accord with the reaction mechanism given above. Additional data are being accumulated which, it is hoped, will further clarify this point.

It is thus seen that the observed differences between the action of alkali on normal and on infected sera can be accounted for satisfactorily by the partial *in vivo* destruction of native serum proteins to molecules of lower molecular weight during experimental type I pneumococcus infection in dogs.

#### SUMMARY

The polarographic protein reaction has been used to study rates of denaturation of serum proteins. No appreciable differences have been found between rates of denaturation of serum from normal and from type I pneumococcus-infected dogs when wetting agents, or pepsin and trypsin are used.

Alkali produces changes in the polarographic rate of denaturation curves, which differ markedly for normal and infected sera. It is suggested that the partial *in vivo* destruction of the serum proteins during infection to molecules of the size of metaproteins, proteoses, and peptones can account satisfactorily for the observed differences.

We gratefully acknowledge the invaluable help and the unstinted cooperation we received in this study from Dr. W. Harry Feinstone of this laboratory. Dr. Feinstone, while actively studying related phases of pneumococcal infection in dogs, kindly took over the culturing of the pneumococci and the actual infection of the dogs, and followed the progress of the disease systematically with x-ray photographs.

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# LABORATORY METHODS

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## GENERAL

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### THE INTRAVENOUS ADMINISTRATION OF OXYGEN\*

EDWIN E. ZIEGLER, M.D., BOSTON, MASS.

THE purpose of this paper is to present a new apparatus which is designed for the carefully controlled intravenous administration of oxygen.

The idea was conceived that anoxemia could be relieved by the intravenous administration of oxygen. It was believed that this method of administration could be made available for those cases in which oxygen therapy, by the usual route, was not adequate.

The intravenous administration of oxygen to patients is not new. It has been reported several times. In the past, however, technical difficulties with the apparatus available prevented the use of this method of oxygen administration.

In this preliminary report is described an apparatus which is now being used for the intravenous administration of oxygen. The results are highly satisfactory and further work, including case reports, will be made the subject of later papers.

In 1902 Mariani<sup>1</sup> injected 120 c.c. of oxygen in forty-five minutes into a dying tuberculous patient (rate of 160 c.c. per hour). He reported that the pulse rate and respiration were improved. The treatment was not repeated. The patient died the next day.

Tunnicliffe and Stebbing<sup>2</sup> in 1916 treated three very ill patients with oxygen intravenously. Their apparatus consisted of a cylinder of "Brinn's oxygen," which contained, they thought, about 1.5 per cent of nitrogen; an "endurance regulator"; pressure tubing; a piece of glass tubing drawn out at the end; a wash bottle and a screw clip on the tubing. The wash bottle contained a solution of 2 per cent sodium carbonate, part of which was blown out by the stream of oxygen and part remained to wash the remaining stream. The amount of oxygen given was estimated by counting the number of bubbles per minute coming through the wash bottle. These authors state that oxygen can be given at the rate of 600 to 1,200 c.c. per hour, that cyanosis and dyspnea are rapidly relieved, and further: "The beneficial results of the intravenous injection of oxygen were certainly more permanent than could apparently be explained by the mere relieving of the cyanosis." The work done with patients fully confirms this observation. Tunnicliffe and Stebbing conclude their paper as follows: "The object of this preliminary note is not to indicate the specific pathological conditions in which the intravenous injection of oxygen should be used, but simply to point out that in those cases in which

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the inhalation of oxygen either cannot be practiced or gives inadequate relief, the intravenous method of oxygen administration, if carefully carried out in accordance with the directions, is available to the clinician and will give therapeutic results."

Singh and Shah,<sup>3</sup> of the Haffkine Institute in Bombay, in 1940 gave oxygen to six patients with severe pulmonary disease. These workers used two different methods. The first consisted of two syringes, moved by screws, working alternately, to pump the oxygen into the vein. There was a pause of five to ten seconds between the actions of the plungers in the syringes. (This allows blood to flow into the end of the needle in the interval when oxygen is not coming through, increasing the danger of clot formation in the needle. Such a clot, if formed, would be dislodged into the venous blood stream when the oxygen flow is resumed.) The second method was similar to that of Tunnicliffe and Stebbing,<sup>2</sup> which they found was hard to control and was apt to produce pulmonary embolism. With the first method they incorporated a manometer to register the venous pressure, which also indicates any stoppage in the flow of oxygen.

Singh and Shah<sup>3</sup> found that the signs and symptoms of pulmonary gas embolism in man are the same as those in animals. They state: "With slight embolism there is a rise in blood pressure and pulse rate; this does not indicate any danger, and the oxygen should not be withheld. With a greater degree of embolism the blood pressure falls, the pulse becomes feeble, and cyanosis increases. The patient complains of a sense of impending dissolution, measiness over the chest, shortness of breath and pain over the precordial region. Oxygen embolism, however, unless carried to extreme is not dangerous." The above authors further state, "all these (six) cases showed clinical improvement for 24 to 48 hours after injection, as indicated by raised blood pressure and improved pulse."

Summarizing their report, Singh and Shah<sup>3</sup> state: "In man about 10 to 20 c.c. of oxygen a minute (600 to 1,200 c.c. per hour) can be administered intravenously; this is about 10 per cent of the basal requirement. This is the only method by which any considerable quantities of oxygen can be administered by an extrapulmonary route. This amount of oxygen does not appear to be considerable, but still a distinct clinical improvement follows its administration."

The work with animals has been more extensive, but it has, on the whole, not been performed carefully enough and has been very misleading for technical reasons, which will be pointed out.

The first workers to inject oxygen intravenously were Nysten<sup>4</sup> in 1811 and Demarquay<sup>5</sup> in 1866. They used dogs.

Gaertner<sup>6</sup> in 1902 also gave oxygen intravenously to dogs. He stated that it did not obstruct the circulation through the lungs. He found bubbles of gas in the right ventricle but never in the left; he contended dangerous symptoms can immediately be relieved by withholding the oxygen. In 1904 Stuerz<sup>7</sup> injected oxygen intravenously in dogs. He recommended intermittent injections and minimized the possibility of embolism. These injections constitute one of the technical faults which have, in the past, led to the abandonment of this form

of therapy. Intermittent injections cause (1) clotting in the needle, or (2) frequent withdrawal of the needle, and as frequent subsequent venipunctures.

More recent experimental work with animals has apparently been more carefully performed, especially with reference to the measurement of the amount of oxygen administered in relation to body weight. The recent work, however, incorporates practically the same technical errors present in the older methods.

Bourne and Smith<sup>8</sup> in 1927 performed experiments with dogs. They state: "It is seen, however, that in amounts (of oxygen) that would provide some hope of the supply of an adequate amount, pulmonary embolism and right heart insufficiency produce an even greater degree of anoxemia than that already in existence." Here lies the cardinal reason for the failure of former investigators to make the intravenous administration of oxygen successful. It has always been *assumed* that it would be necessary to give an amount of oxygen which would approximate the total or basal requirement of the body. This concept we have found to be erroneous. The fact has been overlooked that the body is (even in severe anoxemia) getting *some* oxygen through the lungs.

Bourne and Smith<sup>8</sup> gave dogs from 0.75 c.e. to 3 c.e. of oxygen per kilogram per minute. This corresponds to a rate of 3,150 c.e. to 12,600 c.e. of oxygen per hour for an average 70 kg. man. In contrast, I have obtained favorable results in man by the intravenous administration of from 200 to 1,000 c.e. per hour, and have given oxygen at these rates for periods up to nine hours continuously.

Macdonald Dick<sup>9</sup> in 1939, following the lead of the afore-mentioned workers, gave oxygen to dogs in the femoral vein. He administered 2 to 3 c.e. of oxygen per kilogram of body weight per minute "with safety" and stated that "much larger amounts can be given for short periods." Converting these amounts to values for an average 70 kg. man, we find that they amount to 8,400 c.e. to 12,600 c.e. per hour. Again, too much oxygen was given. In fact, the amount given was ten times that which has been found to be therapeutic.

In the past the failure to obtain beneficial results in a *practical* manner has been due to several factors. Former workers were under the influence of a great curiosity as to the effects of embolism. They made it a point to give so much oxygen that they could observe the effects of severe oxygen embolism.

Animal experimentation as performed up to this time has exhibited several other defects. Most workers with animals have neglected or considered it unnecessary to produce oxygen want. They have not used apparatus which could supply a constant flow of oxygen at the low rate of flow required.

When too much oxygen is administered by the intravenous route, the difficulty is not due to embolism in the ordinary sense. It is due to what is called *tampon*, caused by blood froth in the lungs and right heart. This phenomenon is also called *vapor lock*. These difficulties are no longer of any importance when oxygen is administered intravenously at a constant volume and pressure, each of which is carefully controlled. This is possible with the apparatus described below.

#### THE APPARATUS

Any apparatus used for the intravenous administration of oxygen must meet certain definite requirements. It must be easily regulated. The amount

of oxygen which is delivered must be directly indicated by a manometer scale calibrated in cubic centimeters of oxygen per hour. The volume of oxygen delivered by the apparatus should range between 100 and at least 1,200 c.c. per hour. The flow of oxygen must be continuous and not intermittent. The apparatus must also be so constructed that the flow of oxygen will not be stopped by an increase in the venous pressure. The apparatus consists of the following:

A tank of medicinal grade oxygen, which should be at least 99.5 per cent pure. Such cylinders of oxygen are commercially available. The size of the oxygen tank may be whatever is convenient; a small tank, such as a 20 or 40 gallon tank, would be relatively light, take little space, and could be readily transported.

Attached to the oxygen cylinder by a yoke is a double pressure reducing valve. Attached to the yoke is an indicator showing the pressure of the oxygen in the tank. The first of the pressure reducing valves is a fixed valve requiring no adjustment. This valve reduces the high pressure in the tank to a low level approximating some convenient low pressure, such as 5 pounds per square inch.

The second of the pressure reducing valves through which the oxygen must flow is adjustable. This valve must be so constructed that it will deliver a pressure ranging from zero up to about 200 mm. of mercury, as set. The spring and screw which regulate this valve must be so designed that the pressure can be easily adjusted with an accuracy of 1 to 2 mm. of mercury or better. The outlet from the adjustable pressure reducing valve must have a manometer attached to it to indicate the reduced oxygen pressure. This manometer can be of the mercury type or the so-called aneroid or spring type. Whichever type of manometer is used, it should be calibrated in millimeters of mercury from zero to at least 200 mm., it must also be calibrated to show the flow of oxygen in cubic centimeters per hour.

From the outlet of the second pressure reducing valve the oxygen is conducted by tubing to a filter which contains a convenient number of layers of cotton and calcium chloride or anhydrous calcium sulfate, or some other efficient dehydrating substance. The purpose of this filter is to make sure that the oxygen will be dry. From the filter the oxygen is conducted through more tubing to a small chamber containing a resistor.

This resistor may consist of a porcelain block or a block of some other suitable porous substance, such as porous bronze. The pressure of the oxygen will force it through this porous block. The rate of flow of the oxygen will be directly proportional to the difference in pressure between the oxygen on the proximal side of the porous resistance block and the pressure of the oxygen on the distal side of the porous resistance block, multiplied by a factor which will be constant for any given resistance. This latter factor would have to be determined by the calibration of each apparatus constructed.

Incidentally, the dehydrating filter and the porous fixed resistor would each remove any foreign particulate matter from the oxygen flowing through the apparatus if any was present.

From the housing containing the porous resistor, the oxygen is conducted by tubing to two places. First, the oxygen is conducted back to the mercury or aneroid manometer, but to the opposite side of the manometer, so that the manometer shows directly the difference between the pressures on each side of the fixed resistance. Second, it is conducted through tubing to a wash bottle where the oxygen bubbles through a liquid such as distilled water. This makes the flow of oxygen visible and humidifies it.

From the wash bottle the oxygen is conducted through a suitable outlet to which is attached in each case, before the apparatus is used, a sterile outfit consisting of a sufficient length of rubber tubing, a small two-way valve so that a syringe may be attached to the tubing, a glass adapter, and a needle for injecting the vein. I have adopted a 22 gauge needle.

To use the apparatus the oxygen is turned on and allowed to flow for a reasonable time so as to wash out any residual air. While the oxygen is flowing, the needle is inserted into the patient's vein.

When a tourniquet is used to distend the veins, it causes an appreciable increase in venous pressure. Therefore, when the needle is first injected, and before the tourniquet is released, a small amount of blood may flow into the needle. However, when the tourniquet is released, the oxygen pressure immediately pushes the blood back into the blood stream. There is a small amount of blood remaining which adheres to the surface of the lumen of the needle. This blood may be washed out of the needle by attaching a syringe containing a solution of sterile sodium citrate or other suitable anticoagulant solution of suitable strength to the small two-way valve. The valve is so adjusted that the citrate solution can be injected from the syringe into the rubber tubing just proximal to the glass adapter. The valve is then adjusted to cut off the flow from the syringe and to allow the oxygen to flow through the sterile rubber tubing. The oxygen coming through pushes the sterile citrate solution through the glass adapter and the needle, washing out any blood which may be present. This process of adding citrate solution to the tubing can be used previous to the initial injection of the needle into the vein.

It is also recommended that the apparatus have a manometer, preferably of the aneroid type, with a range from zero to about 60 mm. of mercury. This manometer is attached by tubing to the apparatus at some point between the fixed resistance block and the sterile rubber tubing. It is suggested that it be attached so as to show the pressure of the oxygen in the wash bottle. This pressure will correspond very closely to venous pressure.

Normal venous pressure ranges between 4.4 and 8.8 mm. of mercury. In heart failure venous pressure will rise from about 9 mm. of mercury to as high as 30 mm. of mercury in severe grades. If there is any stoppage in the apparatus, such as would occur if a clot were to form in the intravenous needle, this would be indicated by an unusual rise in pressure, as shown by the gauge. This gauge also provides valuable clinical information as to the degree of heart failure.

The outlet in the wash bottle from which the oxygen bubbles must have a small aperture. If this aperture is large, the bubbles coming through will be large and will cause a "bump effect," which is really a slight variation in the

pressure of oxygen. This effect may cause an uneven flow of oxygen into the vein. The apparatus must be so constructed that the oxygen flow is always uniform.

One should use a new stainless steel needle, entirely free of corrosion, or a gold or platinum needle. See Figs. 1 and 2 for further details.

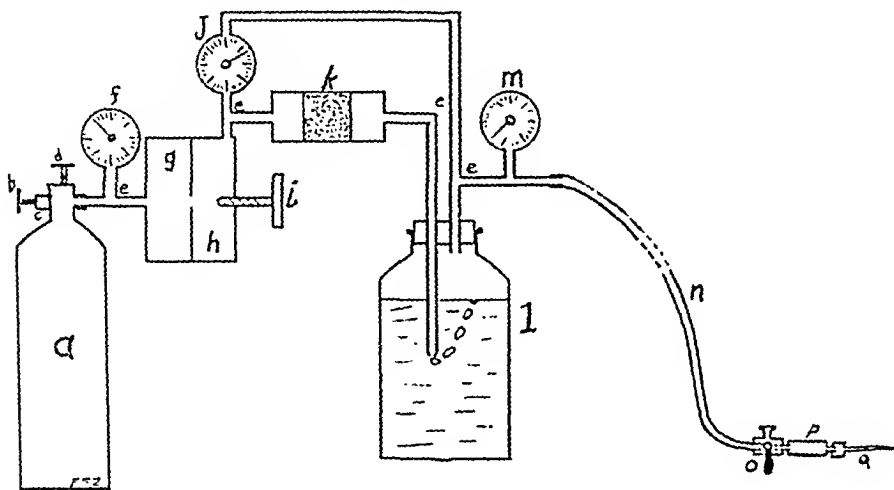


Fig. 1.—Apparatus for intravenous oxygen therapy. Schematic drawing: *a*, tank of compressed oxygen; *b*, screw for fastening yoke to tank; *c*, yoke; *d*, valve for opening tank; *e*, tubing; *f*, gauge showing tank pressure; *g*, fixed pressure-reducing valve; *h*, adjustable low-pressure-reducing valve; *i*, screw for adjusting oxygen pressure and volume of flow; *j*, manometer showing volume of oxygen flow; *k*, fixed porous resistance; *l*, wash bottle; *m*, venous pressure gauge; *n*, *o*, *p*, *q*, sterile tubing, two-way valve, and adapter for receiving glass syringe, glass adapter, and intravenous needle.

The apparatus I have devised always operates under positive pressures of oxygen without interruption or variation, except as adjusted. Any leak developing in this apparatus could not possibly admit air, as oxygen would escape from the apparatus to the outside, preventing air from gaining access to the apparatus.

An additional advantage of this apparatus is that it always supplies oxygen by pressure which is slightly greater than that of the venous pressure. If the venous pressure varies, the pressure in the apparatus will vary accordingly, always remaining slightly higher than the venous pressure. Other types of apparatus that have been used experimentally and that have not been practical for therapeutic work have supplied oxygen at pressures determined by the characteristics of the apparatus without respect to the venous pressure.

Another advantage of the apparatus is that it requires no pump device or electric motor. The oxygen proceeds through the apparatus by virtue of the pressure of the oxygen in the compressed oxygen cylinder. Thus the apparatus can be used at any place, whether or not there is a source of energy, such as electric power.

#### CLINICAL CONSIDERATIONS

Cases will be reported in detail later. A large variety of disease conditions in which oxygen therapy is indicated has not been available up to this time. However, the patients who have been treated have shown in general the following results: Cyanosis is abolished. The pulse rate becomes lower.

The blood pressure, if low, tends to rise. The mental state becomes clearer. One patient who was comatose became conscious within a few minutes. One who had severe pulmonary edema was entirely relieved of the edema after twelve hours, following a five-hour intravenous administration of oxygen.

Various comments have been made voluntarily by patients to the effect that they preferred this form of oxygen therapy to others which they have experienced.

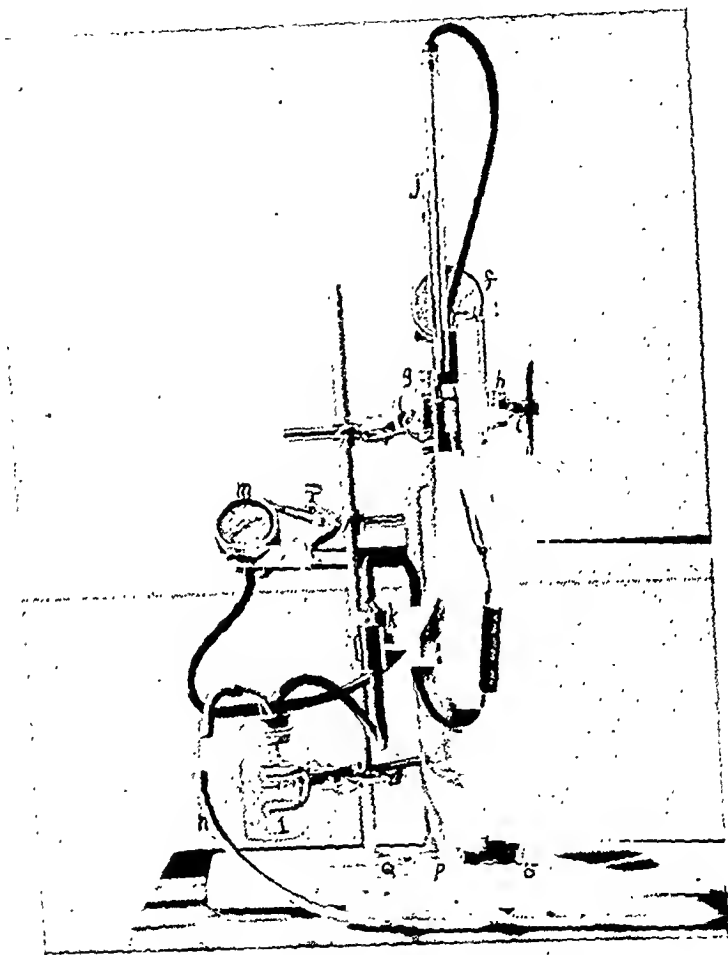


Fig. 2.

The treatment is no more complicated than the administration of solutions intravenously, and the apparatus requires but little more attention. The nursing care of the patient is much easier than when he is in an oxygen tent. No unfavorable reactions, subjective or objective, have been observed thus far.

I have not had the opportunity to study the signs and symptoms of pulmonary oxygen embolism, which others have observed, because I do not give enough oxygen to cause pulmonary embolism. According to Singh and Shah,<sup>3</sup> the signs and symptoms of pulmonary gas embolism (tamponade) are the same as those observed in animals. These authors state, "With slight embolism there is a rise in blood pressure and pulse rate; this does not indicate any

danger; and the oxygen should not be withheld." With this statement I cannot entirely agree. I believe that if an amount of oxygen is being given, which causes a rise in blood pressure with a corresponding rise in pulse rate, the flow of oxygen should be reduced in amount. The signs and symptoms of pulmonary oxygen embolism apparently furnish adequate warning of an overdose.

In my experience, using therapeutic amounts of oxygen, averaging 200 to 600 c.c. per hour, I have observed that when the blood pressure is low, as in shock or heart failure, it will rise. There is also an accompanying slowing of the pulse rate and a less labored and a slower respiration. *Thus the pulse rate and the respiratory rate are the important things to observe during the intravenous administration of oxygen.* An appreciable increase in pulse rate and respiratory rate occurring during the administration of oxygen would indicate that the rate of oxygen administration was too great.

#### ECONOMICS

On a twenty-four-hour basis the cost of the oxygen administered intravenously would not be over ten cents, and would probably be somewhat less. Contrasted to this, the cost of oxygen, on a twenty-four-hour basis, as administered by tent might range anywhere from \$6.00 to \$20.00. The original cost of the apparatus would be much less than the average type of apparatus used at the present time for administering oxygen by other routes.

#### DISCUSSION

It is my belief that the intravenous administration of oxygen is more effective and very much less expensive than other methods of oxygen therapy.

The improvement observed in patients seems to be all out of proportion to the small amount of oxygen administered. This is rather difficult to explain in exact scientific terms. But an explanation is offered as follows:

When oxygen is administered intravenously, the body is getting something that it urgently needs without expending any energy. It requires a certain amount of energy for the pumping action of the heart and the respiratory movements of the chest to obtain oxygen for the use of the tissues. Any oxygen given intravenously reduces the amount of work thus required of the heart and chest. In addition to this, the physiologic processes of the body, in general, seem to be improved, no doubt as a result of improved tissue metabolism.

When oxygen is given intravenously, it provides opportunity for reduced hemoglobin to absorb oxygen directly. This direct absorption of oxygen by hemoglobin can occur in the vein, in the right side of the heart, and finally in the vessels of the lungs. That this absorption does occur is shown by the clinical improvement which results.

If one listens with a stethoscope over the tricuspid area of the chest during the intravenous administration of oxygen, one hears bubbles of oxygen rushing about in the right side of the heart. If one then listens over the mitral area of the chest, no bubbles can be heard. This is an indication that the oxygen is absorbed before it reaches the left side of the heart.



Another possibility remains; this is, that the oxygen, or a portion of it, may not all be absorbed by the venous blood and may pass from the lung capillaries into the alveoli of the lung. On the grounds of clinical evidence I do not believe that this happens. Diek<sup>9</sup> studied this point and states, "it should be noted in this connection that the oxygen consumption of the animal remained unaltered, the oxygen introduced by vein being compensated for by a decreased absorption from the lung." This finding, based on careful work done by Diek, was in spite of the fact that he gave approximately ten times as much oxygen as I have found necessary for therapeutic results.

The clinical evidence of improvement is perhaps not sufficient to convince all of the value of this method of treatment. It is proposed to measure the oxygen consumption with a metabolism apparatus before and during the intravenous administration of oxygen in a manner similar to that used in animal work by Diek.<sup>9</sup> Another laboratory method which I expect to use is the analysis of arterial blood for its oxygen content before and during the intravenous administration of oxygen.

It is believed that the effect of the intravenous administration of oxygen should be determined in certain types of mental disease, such as alcoholism and dementia praecox.

Another brief discussion of oxygen embolism is in order. The phenomena of air embolism are produced by nitrogen. Bubbles of nitrogen present in the capillaries of a tissue will shut off this tissue from its supply of oxygen. In contrast to this, bubbles of oxygen in the capillaries of a tissue cannot shut off this tissue from its supply of oxygen. Animal and human experiments indicate that tissues will gradually absorb these bubbles of oxygen. When this happens, the blood flow through the capillaries is again re-established, and the tissues in the meantime have not been deprived of oxygen.

I have been informed that medicinal grade oxygen is 99.5 to 99.8 per cent pure. The impurities present are said to consist mostly of nitrogen with slight traces of other inert gases. From what I know of the elimination of nitrogen in caisson disease, it can be assumed that this amount of nitrogen is of no significance. In general, those who have employed oxygen intravenously in the past have not had available a supply which was 99.5 per cent pure.

Two contraindications to the intravenous administration of oxygen have been discovered. When there is a marked diminution in the capacity of the vascular bed of the lungs, such as occurs in advanced tuberculosis and emphysema, the amount of oxygen given intravenously should be greatly reduced in amount, or not given at all.

When there is a marked dilatation of the right ventricle, as shown by an unusually high venous pressure, intravenous oxygen may be of no avail. The above conditions are obviously related, since increased resistance to the flow of blood through the lungs is the usual cause of dilatation of the right ventricle.

The apparatus presented may be used experimentally for the intravenous administration of gases other than oxygen, such as gaseous anesthetics, gas-oxygen mixtures, and other gases.

## SUMMARY

An apparatus has been devised and described which makes the intravenous administration of oxygen safe.

The intravenous administration of oxygen to patients has, in my experience thus far, been more effective than oxygen administered by any other routes.

The cost of the administration of oxygen intravenously is almost insignificant in comparison with administration by other methods.

It is believed that the intravenous administration of oxygen can be used in most of the conditions in which oxygen therapy is indicated.

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## CULTIVATION OF *SPIROCHAETA PALLIDA* ON THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING HEN EGG\*

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MANY attempts have been made to cultivate *Spirochaeta pallida* on artificial media.<sup>1-7</sup> At present, however, infected rabbits are the only reliable source of the organisms for experimental purposes. To obtain fairly large quantities of spirochetes more conveniently, and possibly more quickly, we have attempted their cultivation on the chorio-allantoic membrane of the developing egg. Sterzi and Standacher<sup>8</sup> have reported survival of the organisms on the chorio-allantois of the egg for six hours, and retention of virulence for two hours, using the Truffi strain of *S. pallida*.

In our work the Nichols strain† was used directly after one rabbit passage. The material used in the beginning was a frozen 50 per cent testicular mince suspended in 1 per cent neopeptone veal infusion broth, pH 6.9.<sup>9</sup> The mince was thawed rapidly in a 37° C. water bath, and 0.25 c.c. was injected into the right testicle of a healthy male rabbit. The animal showed characteristic lesions of rabbit syphilis thirty-nine days after inoculation. A dark field of the testicular fluid revealed many motile spirochetes. The rabbit was sacrificed by injecting 20 c.c. of air into the external vein of the left ear. The testicle

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†This material was kindly furnished by Dr. W. L. Fleming, of the Division of Public Health, University of North Carolina.

was excised, cut into pieces of about 5 c.mm., and ground for ten minutes with sterile sand. One per cent Difco peptone beef infusion broth, pH 7.4, was used to make a 50 per cent suspension by weight of the testicular material. The suspension was well mixed, and the sand and heavy tissue were thrown down by centrifuging at low speed (350 r.p.m.) for five minutes.



Fig. 1.—Rabbit No. 15, fifty-nine days after inoculation.

Using the Goodpasture technique,<sup>10</sup> 0.1 c.c. of the supernatant fluid was dropped onto the chorio-allantoic membrane of each of ten fertile hen eggs which had been incubated ten days at 38° C. After inoculation the eggs were returned to the 38° C. incubator. The time elapsed from the rabbit's death to inoculation of the eggs was one and one-half hours.

One of the embryos ceased normal movement seven hours after inoculation. At this time, 0.25 c.c. of its chorio-allantoic fluid was injected into each testicle of a healthy male rabbit (No. 14). The material injected showed a few feebly motile spirochetes by dark-field examination. However, no syphilitic lesions have been observed in the rabbit in ninety days.

A second embryo was dead twenty-seven hours after inoculation. This egg was opened, and 0.25 c.c. of its chorio-allantoic fluid was injected into each testicle of another healthy male rabbit (No. 15). Dark-field examination showed one or two sluggishly motile spirochetes per field. Forty-seven days after inoculation the right testicle of this animal was swollen and indurated, the left testicle remaining normal in appearance. Fifty-nine days after inoculation both testicles were greatly swollen and hard masses could be felt. There was a chancre-like lesion 2 cm. in diameter on the left testicle (Fig. 1), and numerous motile spirochetes could be seen in dark-field preparations.

On the sixty-fourth day following inoculation, rabbit No. 15 was sacrificed; both testicles were cut and ground with sterile sand, then mixed with Difco peptone beef infusion broth, pH 7.4, to make a 50 per cent emulsion by weight. The supernatant liquid was prepared by centrifuging as before, and 0.5 c.c. of it was used to inoculate another rabbit (No. 19). At this time, the preparation showed many motile spirochetes by dark-field examination. The remainder was frozen and preserved at  $-78^{\circ}$  C.

Tissue sections of the liver and heart of the embryo from the egg used to inoculate rabbit No. 15, stained by Levaditi's method, showed no spirochetes. The remaining eight of the ten eggs inoculated were allowed to hatch, and the chicks appeared normal for ten days, the limit of observation. Tissue sections of their hearts and livers taken at this time showed no recognizable spirochetes.

Twenty-four days after inoculation rabbit No. 19 showed swelling and induration of both testicles. Thirty days after inoculation dark-field preparations of material aspirated from each testicle showed numerous motile spirochetes.

The infection of rabbit No. 15 indicated that virulent spirochetes of the Nichols strain will live twenty-seven hours in the developing hen egg and, in some cases, will retain their virulence for that length of time. The infection is transferable from one rabbit to another, the first rabbit-to-rabbit transfer producing rabbit syphilis within the normal time limit. More work is necessary to determine how great a percentage of the egg inocula will live, whether spirochetes will multiply in the developing egg, and in which part of the egg surviving or growing spirochetes are most abundant.

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## TENSILE STRENGTH OF HAIR AND HAIR ROOTS

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IN THE preparation of animals for intradermal or subcutaneous injections, the usual practice is to shave the dry or lathered hair with a razor, sometimes after preliminary clipping with a scissors or hand or electric clipper.<sup>1</sup> Occasionally a chemical depilatory, such as barium sulfide, is used. At the Olive View Sanatorium it has been found simpler to pull out the hair from the guinea pigs in about half a dozen tufts, thus clearing a space large enough for the injection. This plucking is faster, lasts longer, and produces less local irritation. Only a few seconds are required, as compared with the longer time needed for clipping, lathering, shaving, and getting, cleaning, and replacing the clippers and razors, or applying and removing the depilatory. The part which is plucked remains bare for many days longer than the part which is shaved, thus allowing better observation of local reactions that may occur. With the plucking of hair, the guinea pig skin is left smooth and pale, and no inflammatory reaction follows, so that the reaction to injections may be readily noted. On the other hand, shaving is not only followed by gross cuts and injuries, but microscopic examination of the freshly shaven skin indicates that many subvisible lesions may also be inflicted, any of which increases the chances for local irritation, inflammation, and infection.

Insofar as sensation or distress can be gauged by the behavior of the animals, it appears that plucking is less painful to the guinea pigs than the more prolonged procedures of shaving or applications of epilating pastes. The guinea pig hair usually comes out so readily that there is not the tension on the skin, which is the usual cause for pain in hair-pulling. Guinea pigs seem much more frightened than hurt by the procedures in general, and soon become used to handling. They usually utter the greatest amount of audible complaints, squeals, etc., while they are being sought and picked up, subsiding soon after they are firmly grasped. If not too great a tuft of hair is taken at each time, and the plucking is done rather brusquely, they may make no further protest during the entire procedure, or at most only a single yelp at the first touch. With a little practice technicians become so adept at this mode of epilation that they can prepare a large number of animals for testing within a few minutes, with practically no complaint on the part of the subjects.

The ease with which hair may be plucked from guinea pigs is markedly in contrast with the resistance to such hair-pulling shown by rabbits and other animals, especially the fur-bearing species. For such subjects, shaving offers a much better means of baring the skin. Very simple observations were suf-

ficient to demonstrate that this was the case. Even the removal of a few hairs from a rabbit is difficult; to pluck a large clump of hair may result, if sufficient force is used, in actual disruption of the skin, if the hair does not, as is more often the case, break in the grasp.

In order to obtain quantitative data regarding the ease of removal of hair from the guinea pig, a series of simple measurements was undertaken. A light wire spring scale was calibrated by attaching various small weights, from 1 to 100 Gm., to a mosquito forceps at its lower end. When the forceps grasped a hair instead, and the spring scale was withdrawn, the elongation of the spring and the corresponding tension exerted were readily observed.

Ordinary human hair, pulled from the forearm of an adult male, exerted a tension of from 5 to 25 Gm. before extraction, with an average of 12 Gm. per hair. Thereafter, pulling at the extracted hair until it broke off somewhere in its length required another force of from 8 to 32 Gm., with an average of 18 Gm. per hair.

Accordingly, a human hair will usually, when pulled sufficiently, come out from the skin, but if jerked rapidly, it is apt to break off, since the breaking stress is about 50 per cent greater than that of epilation.

On the other hand, hair from the back of a guinea pig came out with the application of from 1 to 5 Gm. force, usually about 2 Gm. However, the same hair could not then be broken without the application of from 10 to 52 Gm. of tension, averaging about 25 Gm. It appears, then, that the hair of the guinea pig is really somewhat stronger than that of man, but that the hair root in the guinea pig is quite fragile, releasing the hair at less than one-sixth the tension required to pull out a human hair, and less than one-twelfth the force required to break off the hair. Accordingly, the chances of breaking off the hair on plucking a guinea pig, instead of extracting it, are very slight.

Further studies in the same manner may be desirable to elucidate the effect of season, sex, age, pregnancy, hair, and skin color, hair length and thickness, curliness of hair, etc., as well as the effects of various diets, diseases, and environmental factors on the hair of different species.<sup>2</sup>

The present study merely documents the previously observed fact that the hair of the common guinea pig is readily removed by simply plucking it out in clumps, and that this makes a much simpler and more effective way of epilating a guinea pig than does shaving, clipping, or the application of depilatory paste or lotions.

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## AN INEXPENSIVE ANIMAL COMPRESSION CHAMBER\*

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IT IS possible to construct a simple and efficient compression chamber for small animals at very low cost. The chamber described here was built in the school shop at a cost of less than \$15.00 and has given almost uninterrupted service for four years. It was originally used in animal experiments incidental to the development of helium-oxygen mixtures for use by divers and others working under increased atmospheric pressure.<sup>1</sup> It can be used to subject rats, mice, guinea pigs, and small rabbits to increased pressure in any gas or mixture of gases. By substituting an appropriate gauge and using suction instead of high pressure, it may be employed in experiments in reduced pressures as well.

The body of the chamber consists of a 1-foot section of 6 inch standard steel pipe, carefully squared on the ends. Next, a circular steel plate 10 inches in diameter and  $\frac{5}{8}$  inch in thickness is converted into the door or back cover of the chamber. This plate is centered in a lathe, and a shallow, circular trough is cut into one face. The trough is just wide enough to accommodate the end of the 6 inch pipe comfortably. Later this trough is filled with melted lead to form a gasket. The center is then cut out of a second plate of the same size. The ring thus produced is just large enough to slide over the outside of the chamber and form a collar for it. This collar is later welded in place 1 inch from the back end of the chamber. A third plate of the same size has a 4 inch circle cut from its center, with the cut beveled  $\frac{3}{4}$  inch toward the outside. This last plate covers the window of the chamber and holds it tightly in place. The window itself consists of a  $6\frac{3}{4}$  inch circle of plate glass 1 inch in thickness.

The various parts of the compression chamber are held together by eight steel rods made from eight 16 inch lengths of  $\frac{3}{4}$  inch mild steel rod, threaded for a distance of  $1\frac{1}{2}$  inches on one end and for  $3\frac{1}{2}$  inches on the other. These rods pass through holes drilled in the collar and end plates of the chamber. First the two end plates are drilled with eight  $\frac{13}{16}$  inch holes spaced evenly around the margin, with their centers  $\frac{7}{8}$  inch from the edge. Using these plates as a template, the collar is then drilled with eight holes which are tapped to take the threaded rods. The rods are all threaded through the collar from one side, using the ends which had been threaded for a distance of  $3\frac{1}{2}$  inches. The collar is then easily held in position while being welded to the chamber by placing the two end plates in position over the ends of the rods and drawing them up snugly by means of nuts.

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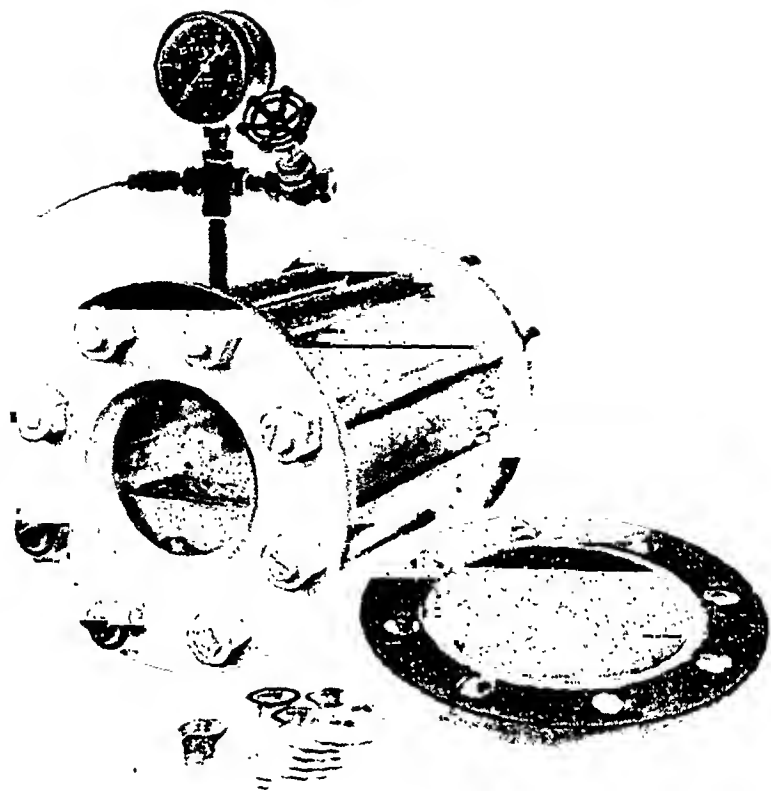


Fig. 1.—Compression chamber showing view through window; method of attaching gauge and valve; and back cover removed to show lead gasket.

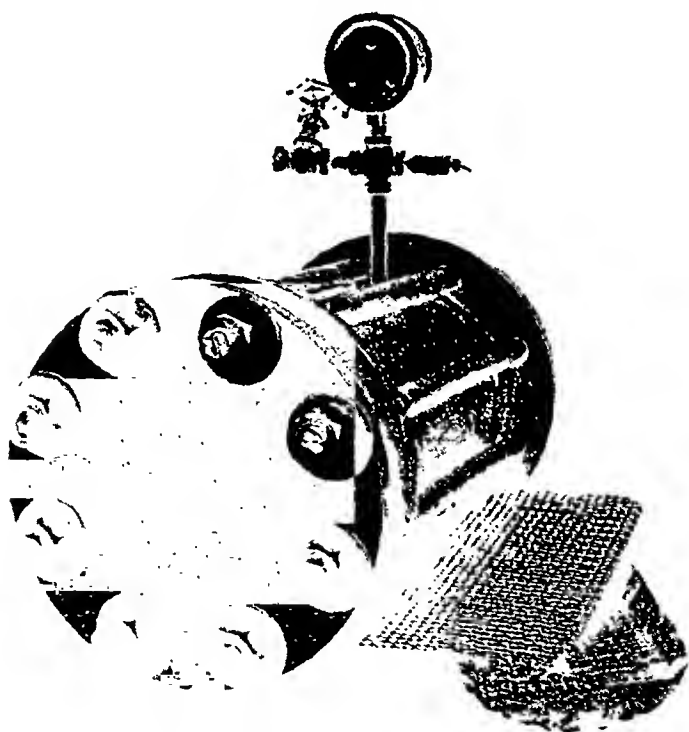


Fig. 2.—Back view of chamber with cover in place. The tray for chemicals and the screen floor of the chamber are shown.



The chamber is assembled by placing a thin, paint-soaked leather gasket between the end of the pipe and the window, and a plain gasket between the window and the plate which covers it. This plate is then drawn up very tightly and evenly by means of nuts on the ends of the  $\frac{3}{4}$  inch rods. The cover of the chamber is tightened in place in the same way. The cover and one of the rods are punch marked to insure replacing the cover in the same position every time. After the nuts have been tightened and released several times, the lead gasket becomes shaped to the end of the pipe and forms a tight joint when the cover is drawn up tightly and evenly.

The chamber is completed by drilling a hole through the top and threading into it a short piece of heavy  $\frac{1}{2}$  inch brass pipe, to which is attached a brass cross. A pressure gauge is then attached to the vertical arm of the cross, and a needle valve to each side arm. Such an arrangement permits the introduction of gas through one side arm and its subsequent removal through the other one. Also, two gases or mixtures of gases can be used by introducing one from each side. As can be seen from Figs. 1 and 2, a packing box has recently been substituted for one of the needle valves to permit an insulated wire to be run into the chamber to provide current for illumination whenever it is desired to photograph the animals.

Carbon dioxide and moisture may be eliminated by ventilating the chamber; a galvanized iron tray of chemicals beneath the screen floor assists in this elimination. The bottom of this tray is filled with calcium chloride to absorb moisture. A blotter saturated with concentrated sodium hydroxide solution, to absorb excess carbon dioxide, is supported on a screen above the tray. The floor of the chamber is made of heavy screen placed high enough above the blotter to prevent any caustic from coming in contact with the animals.

The chamber before being used for experiments should be tested by filling it with water and then subjecting it to a pressure considerably higher than any that will be used experimentally. A cylinder of compressed air provides a very convenient source of high pressure for this test. Leaks will be betrayed by the escape of water, and no violent explosion will occur if any part breaks. It is theoretically safe to employ pressures of several hundred pounds per square inch in this chamber, but we have never had occasion to use pressures in excess of 100 pounds. If high pressures are used, a mirror should be arranged to permit the observer to see the inside of the chamber without standing in front of the glass window itself.

#### SUMMARY

The construction of an experimental compression chamber at a cost of less than \$15.00 is described. In this chamber rats, mice, guinea pigs, and small rabbits can be subjected to pressures in excess of 100 pounds per square inch in any gas or mixture of gases, and their reactions can be observed. The chamber may be employed in studying the effects of reduced pressure.

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# A DIRECT PLETHYSMOGRAPHIC METHOD FOR DETERMINING THE BLOOD PRESSURE IN THE UNANESTHETIZED RAT\*

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WITH the development of techniques for producing hypertension in the rat, many investigators have attempted to devise methods for the indirect measurement of blood pressure in small rodents. Early methods made use of anesthetized animals,<sup>1</sup> but since the anesthetic itself had some effect on the blood pressure, methods were sought for use on the nonanesthetized animal. The first of these was that of Diaz and Levy,<sup>2</sup> which gave reliable results but necessitated the cutting of the tail and some loss of blood. Williams, Harrison, and Grollman designed an ingenious apparatus which, in their hands, gave very consistent results.<sup>3</sup> In the course of our investigations this method was used with some success, but we encountered several difficulties. In the first place, the method requires that the animals be warmed and trained, and is not always feasible. Furthermore, there is no objective criterion by which to determine whether the animals have been equally warmed. In addition, we found that since the tail is separated from the water in the water chamber by a surrounding thin rubber tube, the apparatus is not very sensitive to small variations in pressure, and works best with a rat whose tail fits close to the rubber tube. In the case of small rats it is not always possible to collapse the tube about the tail without leaving air spaces, and as a result the expansion of the tail at systolic pressure may be taken up by the slack of the rubber tube.

To obviate these difficulties we designed the following apparatus which employs direct plethysmography, and requires neither training nor preliminary heating. In our hands this apparatus has given very consistent results.

## DESCRIPTION OF THE APPARATUS (Fig. 1.)

Part 1 consists of a cylinder 2 inches in diameter and 6 inches long, made of continuously perforated tin or other metal. This is closed off at *a* by a cork which can be used to adjust the tube length. The other end is closed by a sleeve cylinder of metal made to fit over the tube (about  $2\frac{1}{8}$  inches in diameter). This sleeve is completely closed at *b* except for a  $\frac{3}{8}$  inch hole drilled in the center to permit passage of the tail. This part holds the animal.

Part 2 consists of a glass cylinder of 1 inch diameter and  $\frac{1}{2}$  inch long, closed at both ends except for the flanged apertures at *c* and *d* as shown. These are  $\frac{3}{8}$  inch and  $\frac{7}{16}$  inch, respectively. At *e* there is a short glass tube of  $\frac{1}{16}$  inch bore to admit air. Cigarette drainage tubing of  $\frac{3}{8}$  inch diameter is passed through the two apertures, stretched and cemented over the flanges, and tied down. This completes the pressure cuff. For small rats of less than 100 Gm. a similar cuff is used, but with apertures of  $\frac{1}{4}$  inch and  $\frac{3}{16}$  inch, respectively.

\*From the Department of Anatomy, McGill University, Montreal.  
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Part 3 is a cylinder of glass,  $\frac{3}{4}$  inch in diameter, with a flanged opening of  $\frac{1}{4}$  inch at *f*, and closed at *g*. The tube length is 6 inches. At the superior surface of the distal end an S-shaped capillary manometer (0.5 mm.) is placed, which rises 2 inches above the tube. At *h* and *i* stopcocks are placed for water inlet and outlet. A one inch length of  $\frac{1}{4}$  inch cigarette drainage tubing is cemented in place over the flange, which when snugly applied to the tail acts as a stopper for that end of the tube. This diameter of tubing is most satisfactory for overall use, while  $\frac{5}{16}$  inch will be found better for larger animals (above 150 Gm.) and  $\frac{3}{16}$  inch for small animals (less than 100 Gm.). The design of the apparatus permits replacing or changing of the rubber tubing in a few minutes.

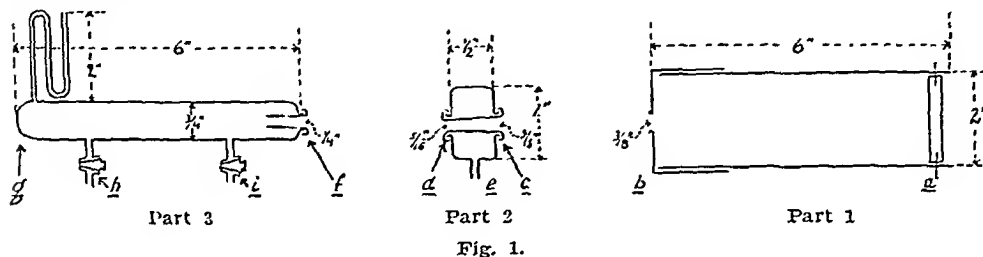


Fig. 1.

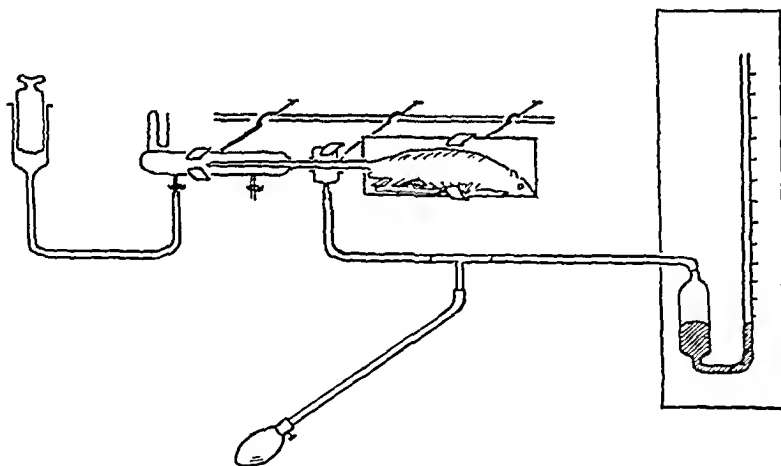


Fig. 2.

The general arrangement is shown in Fig. 2. The three parts are held together with clamps that slide on a rod. The pressure cuff is connected both to a mercury manometer and to a bulb with a shut-off valve, via a glass two-way connection. The water chamber is connected to a 50 c.c. syringe which acts as a water reservoir and allows the same water to be used without continually replenishing a simple water reservoir. The entire apparatus may be conveniently housed in a three-sided box.

The rat is gently placed in the holder that is then closed with the covering sleeve, allowing the entire length of the tail to protrude freely. The holder is placed in the clamp and the animal is allowed to sit for a minute to accustom itself to the holder. If the animal is very restless, an electric light placed beside the holder will usually give it a little warmth and quiet it. The tail

is then passed through the pressure cuff which is moved, together with its clamp, as close to the holder as possible. Soft vaseline is smeared on the distal end of the tail for about one inch from the cuff, and the tail passed through the sleeve into the water chamber. This latter is moved as close to the cuff as possible, so that a maximum of tail will be immersed in water. However, care must be taken that although the rubber sleeve is tight enough to prevent water leaking out around the tail, it is not tight enough to occlude the blood supply to the distal part of the tail. Such an adjustment is in practice quite simple.

Pressure is applied to the tail to a level of 30 to 50 mm. Hg above the expected systolic level. Water is run in from the syringe via the stopcock, filling the water chamber completely, and rising until the manometer is about half filled. Adjustment of the level in the manometer is most easily carried out by withdrawing water through the outlet stopcock. Pressure is then gradually released.

At systolic blood pressure blood will flow into the tail, and—being unable to return through the still occluded venous channels—will cause the tail to expand and the water to rise in the manometer. This expansion will be represented in the manometer by a rise of 2 cm. to 12 cm. or more. With practice it is possible to distinguish accurately two rates of expansion. The first occurs just at systolic pressure and is very slow so that the water level rises in small jerks. The second occurs 15 to 20 mm. below this and is rapid and fairly smooth. The first readings the beginner will make is this lower one, since it is more obvious, but with a little care the higher first point will be distinguished. It is our impression that the lower point corresponds to the unobstructed inflow of blood at diastolic, and hence that our two points probably correspond to those described by Diaz and Levy.

The following difficulties may be encountered. The animal, although not necessarily excited, may move its tail about. Such tail movements are readily distinguished from the true expansion by their jerky character, the large rise of water in the manometer, and the quick fall back again. If a tail movement occurs at an inappropriate moment, pressure is merely released and re-applied a second time. This may be repeated until one is certain of the reading. Another point of caution is that the water chamber should not contain any air, although one or two small bubbles will not interfere with the recording.

The method requires some practice but will, with care, give reliable and consistent results, both for single or repeated pressures, and requires very little time.

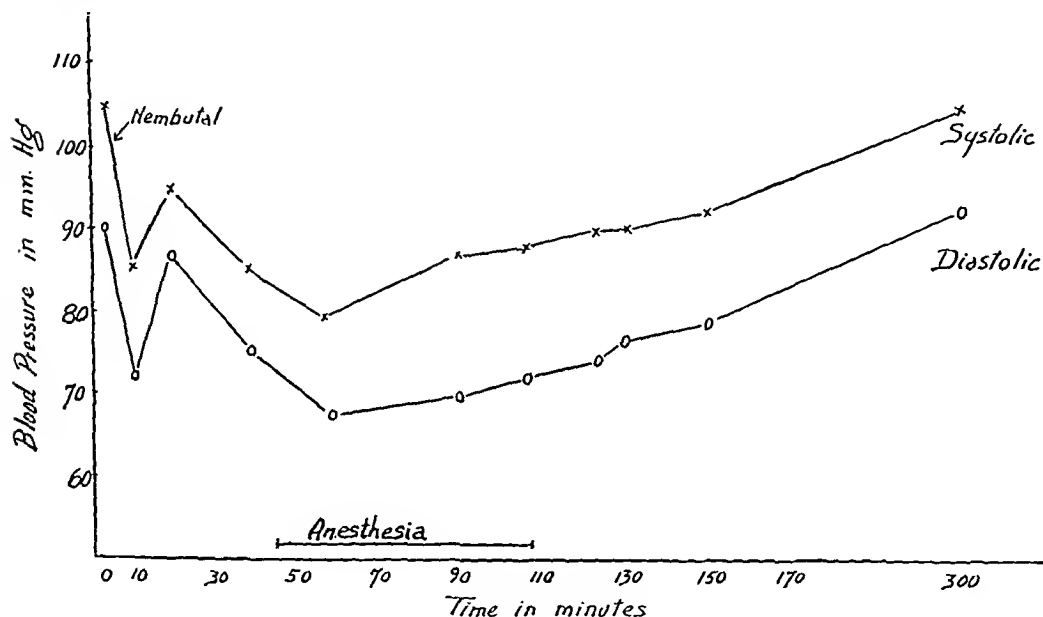
TABLE I

| ANIMAL NO.  | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Body weight | 108 | 129 | 112 | 130 | 116 | 110 | 129 | 109 | 118 | 112 | 128 | 102 |
| Systolic    | 104 | 108 | 108 | 108 | 106 | 110 | 110 | 108 | 110 | 108 | 104 | 104 |
| B.P.        |     |     |     |     |     |     |     |     |     |     |     |     |

We record in Table I the systolic pressure obtained on 12 normal rats with an average body weight of 124 Gm. The consistency of the pressure levels in these animals is striking and agrees well with the results obtained by direct manometry.<sup>4</sup>

In Graph I we record as an example the continuous systolic and diastolic blood pressures of a rat given a small dose of nembutal (sodium pentobarbital) for other purposes.

The normal pressure of the animal was first obtained, the injection was given, and within five minutes the animal was back in the holder and remained there until completely recovered. We wish to draw attention to the fact that more than half the points on the graph were plotted without the animal being under anesthesia, and to observe that diastolic readings represent the second point discussed in the foregoing description.



Graph 1.

## SUMMARY AND CONCLUSIONS

1. A method for the determination of both systolic and diastolic pressures in the nonanesthetized rat is described.
2. Sample records are given to show the magnitude of the results obtained.

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# AN AUTOMATIC QUADRUPLÉ PIPETTING MACHINE FOR THE RAPID AND ACCURATE DELIVERY OF MEASURED SMALL AMOUNTS OF FLUID\*

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A NUMBER of automatic pipettes have been constructed for the delivery of accurately measured quantities of fluids in such tests as the complement fixation test for syphilis. The assemblies of Lorenz<sup>1</sup> and Laird<sup>2</sup> share the common and significant disadvantage of variation, and therefore, inaccuracy in delivery of fluids due to actuation of the two-way valve by the flow of fluid through it. The automatic pipette of Hartman,<sup>3</sup> incorporating a mechanically operated two-way valve, is sound in principle and accurate in operation, but the design of its construction does not readily lend itself to adaptation in a test like the New York State quantitative complement fixation test<sup>4</sup> for syphilis.

The desirability of an apparatus to dispense accurately and rapidly various fluid reagents in amounts ranging from 0.05 c.c. to 0.2 c.c. in the New York State quantitative complement fixation test for syphilis led to the development of an automatic quadruple pipetting machine of high accuracy and precision. Simple in construction and operation, it demands little or no manipulative skill. Apart from this test, the instrument is of value in any routine method, chemical or serologic in character, requiring the repeated delivery of measured small amounts of different fluids. The prime advantages of its design and construction lie in the elimination of complex gearing, belting, and rheostats, thereby avoiding the frequent breakdowns and changes in speed of delivery accompanying their use.

The instrument (Fig. 1) is driven by a  $\frac{1}{20}$  H. P., A. C., 110 volt-60 cycle General Electric small synchronous motor, rotating at an absolutely constant speed of 75 r.p.m. From actual experience it has been determined that the optimum working speed for the average technician is 70 to 90 r.p.m., corresponding to an identical number of deliveries of fluid reagent. Hence, adjustment of the speed of the motor is not necessary. The synchronous motor provides a constant speed and eliminates all electrical regulating devices.

## DESCRIPTION OF APPARATUS (Fig. 1)

An adjustable eccentric (*a*) fastened securely and directly to the shaft of the motor converts the rotary motion into a reciprocating one through a Scotch yoke (*b*), held rigidly by side gibs (*c*) which can be adjusted for take-up due to their wear.

\*From the Eugene Littauer Memorial and Fulton County Laboratory, Nathan Littauer Hospital, Gloversville.

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This eccentric (*a*) moves the pistons (*d*) of the syringes (*e*) in an upward stroke, and at the same time compresses the springs (*f*) which are placed in such a position that the pistons are held firmly against the reciprocating bar (*g*).

Noncorrosive heads (*h*), enclosing double-check valves of a ball-seating fully automatic gravity type, are connected directly to the Luer-Lok syringes. It has been found that this is the most simple and efficient design for the purpose of preventing the passage of fluid in either direction until demanded by the reciprocating stroke of the plunger. The valve is a simple and time-proved device, consisting of a ball seat and a truly spherical ball. The ball seat is angular. The weight of the ball sits in its angularity with a very narrow rim surface, thereby eliminating almost completely any danger of accumulation of foreign matter in the flowing fluids which would interfere with the function of the valve. The two-way valve is actuated by the mechanical action of the syringe plunger upon it with almost complete independence of the flow of the fluid reagents.

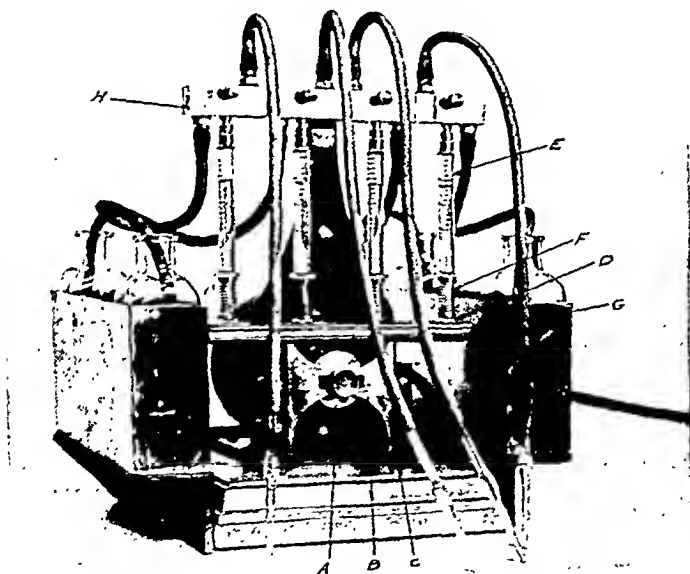


Fig. 1.

To the noncorrosive heads are attached inert rubber tubes of 2 mm. thickness in order to eliminate any pulsations leading to collapse of their walls. These, if present, represent a significant source of error in delivery.

Glass nozzles, prepared from carefully selected 1.0 c.c. standard Ostwald pipettes, are inserted into the outlet rubber tubes. It is manifest that each tube must deliver a constantly uniform volume of fluid without accumulation of a variably sized hanging drop about its opening during, or subsequent to, delivery. This was eliminated by altering the tip and opening the nozzle of the tube, and by polishing its end on a very fine oil stone, thereby eliminating the need of dipping it into oil. Failure to observe these precautions will lead to unequal and, therefore, inaccurate deliveries.

## ACCURACY OF DELIVERY BY MACHINE

The accuracy of the pipetting machine was determined by weighing 10 successive deliveries of 1.0 c.c. water from each of four syringes. Table I shows the recorded weights of these deliveries at a constant temperature of 20° C., as determined on a Christian-Becker chain-o-matic balance. These figures indicate that the individual delivery from each pipette did not vary more than two parts per thousand, thus indicating the very high degree of each individual delivery with the pipetting machine. If even greater accuracy in the deliveries of all syringes is desired, four syringes of exact dimensions must be employed. These can be obtained only by selection from a large number of syringes by trial weighings of individual deliveries under standard conditions.

TABLE I  
WEIGHT IN GRAMS OF SUCCESSIVE DELIVERIES OF SYRINGES

|                | SYRINGE 1 | SYRINGE 2 | SYRINGE 3 | SYRINGE 4 |
|----------------|-----------|-----------|-----------|-----------|
|                | 1.0048    | 1.0069    | 1.0024    | 0.9989    |
|                | 1.0010    | 1.0025    | 1.0007    | 0.9973    |
|                | 1.0012    | 1.0054    | 1.0024    | 0.9980    |
|                | 1.0025    | 1.0042    | 1.0000    | 0.9965    |
|                | 1.0045    | 1.0035    | 1.0035    | 0.9964    |
|                | 1.0049    | 1.0025    | 1.0000    | 0.9971    |
|                | 1.0018    | 1.0023    | 1.0011    | 1.0009    |
|                | 1.0002    | 1.0050    | 1.0000    | 0.9998    |
|                | 1.0050    | 1.0027    | 1.0015    | 1.0019    |
|                | 1.0033    | 1.0029    | 1.0009    | 0.9988    |
| Average weight | 1.0035    | 1.0039    | 1.0012    | 0.9985    |
| Mean deviation | 0.00156   | 0.00129   | 0.00095   | 0.00150   |

Extensive experiments with pipetting by hand carried out under conditions identical with those for the test of the accuracy of the pipetting machine show that the maximum error is in the second significant place and lies between 0.9372 and 0.9982. This maximum percentage error of six parts per hundred, or 6 per cent, compares unfavorably with the maximum deviation of two parts per thousand, or 0.2 per cent, obtained with the automatic pipetting machine. This may be a significant source of error in that, with the pipetting of complement and antigen by hand in the New York State quantitative complement fixation test for syphilis, the possible cumulative error may rise to a total of 12 per cent. It is also of interest to note that all deliveries by hand were below 1.0 c.c., for which the pipettes were standardized. In the actual performance of the test, this would represent a second significant source of error in that smaller amounts of reagent are added than are required, thereby increasing the experimental error.

## CARE OF THE MACHINES

The normal use of the automatic quadruple pipetting machine requires only a general lubrication of its moving parts when necessary, and flushing of the syringes and rubber tubing system with distilled water. The intake tube is immersed in distilled water, and by inserting a spatula beneath the plunger of the syringe and moving it up and down for about 20 full strokes, the



system is thoroughly flushed out. The intake tube is now removed from the distilled water, and with a few additional strokes of the plunger of the syringe, the system is emptied. The valve block need not be disassembled unless there is accumulation of foreign material on the valve seats, but this is extremely uncommon.

#### ADVANTAGES OF AUTOMATIC QUADRUPLE PIPETTING MACHINE

The advantages of the pipetting machine consist in the elimination of all adjustments after its first setting, increased speed and higher precision in the delivery of measured small amounts of fluid reagents, and absence of fatigue of the operator in comparison with pipetting by hand. In addition, there is a complete elimination of breakage and cleansing of serologic pipettes. The time required for the delivery of antigen and complement in various dilutions, salt solution, and sensitized sheep red blood cells in the New York State quantitative complement fixation test for syphilis is considerably reduced and accomplished with increased accuracy.

#### SUMMARY

An automatic quadruple pipetting machine for the accurate and rapid dispensing of liquids in amount of 0.05 c.c. to 0.2 c.c. in the New York State quantitative complement fixation test for syphilis is described. Its operation for over one year in our laboratory has given eminent satisfaction. The instrument can be readily adapted to other laboratory methods. For the delivery of larger amounts of fluid, larger syringes may be substituted.

The machine is manufactured by the Curtin-Hebert Co., Inc., Gloversville, N. Y.

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# THE DETERMINATION OF SENSITIVITY TO PAIN

## A SIMPLE CLINICAL METHOD

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ALL who have read Emanuel Libman's<sup>1</sup> classical essay on the individual sensitiveness to pain must realize that without an appraisal of a patient's susceptibility to pain, there cannot be adequate diagnosis, prognosis, or treatment. The hyposensitive patient's complaints must be magnified in order to obtain a proper perspective. According to Libman,<sup>1</sup> a hyposensitive patient feels less pain, and the pain that he does feel has an unusual radiation. He may have substitution symptoms, e.g., pressure, burning, or numbness instead of pain. Every clinician can probably recall a case or two of a very acute surgical abdomen, where the surgeon was not consulted early because the patient did not have real pain, but had instead burning, pressure, or numbness over the abdomen. Libman believes that in the case of the hyposensitive patient, the pain impulses are delayed in the autonomic nervous system, while in the case of the hypersensitive patient, the impulse travels more directly to the central nervous system. He thus explains the occurrence of yawning, coughing, choking, and sneezing instead of pain in the hyposensitive patient.

There are many methods for measuring the intensity of the stimulus required to cause pain in the skin of a person. It is naturally assumed that the induced pain acts in the same way as a spontaneous pain. Roughly, the methods for inducing this stimulus are as follows: thermal, electrical, chemical, and mechanical. The thermal method was first used by Goldscheider in 1884, and has recently been made extremely accurate by Hardy, Woolf, and Goodell.<sup>2</sup> The drawback is the extensive equipment and the time necessary to determine the pain threshold of a person. It thus will have limited use except in a physiologic laboratory. The excellent papers of Hardy and co-workers show how well this method can be used on a small scale. The electrical method consists of stimulation of the skin with a faradic current and measuring the amount that will cause pain in a person. The exponents of this method who have done important work are Helmholtz, Martin,<sup>3</sup> Macht, Hauck and Neuert. The chemical method has been inadequately studied, possibly because stimulation by this method might cause an actual change in the skin. The mechanical method has been studied by von Frey, Eddy,<sup>4</sup> and others. Libman's test is an example of this method. In this test the examiner's thumb is pressed against the styloid process of the patient. This allows for a rough estimation of the person's sensitivity to pain. It can easily be seen that one factor in this test would be the strength of the examiner. There is, furthermore, an occasional difference between styloid processes. Recently Hollander<sup>5</sup> suggested an ingenious

instrument that consists of a grater sewed to a blood pressure cuff. The cuff is applied in the usual way to the patient's arm and the cuff is inflated. The end point is reached when the patient cries out or winces. According to the most recent work,<sup>6</sup> this method is not considered accurate in obese or in moderately obese patients, and in those with severe or moderate hypertension. These restrictions limit the usefulness.

The instrument used in this study, the sensometer, is also a mechanical method of inducing pain in the skin. The one common drawback of the mechanical method is that the deformation and internal stresses of the skin and underlying tissues are not taken into account. In the method herein described, a part of the body is chosen that is not obese; it is felt that this reduces a serious drawback to the pressure method.

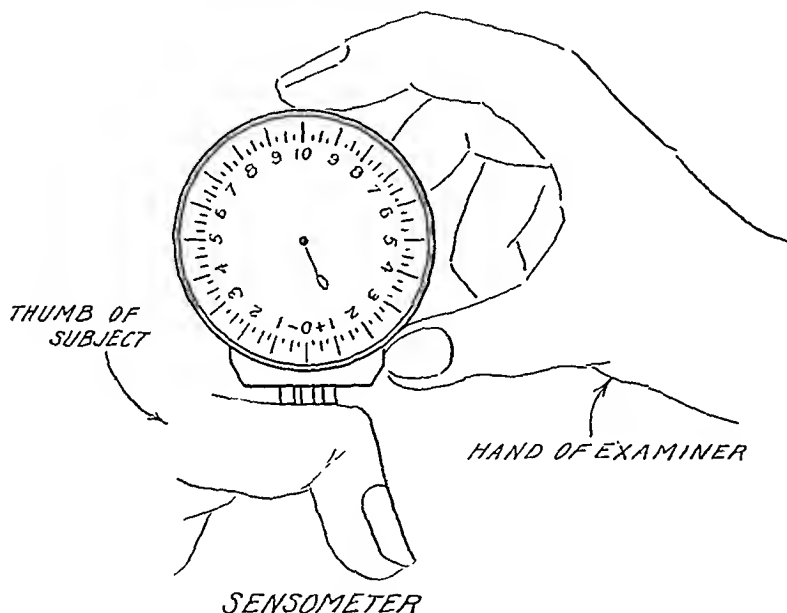


Fig. 1.

#### A NEW METHOD OF ESTIMATING PAIN SENSITIVITY

The device used in this study can be called a sensometer (Fig. 1). It is adapted easily from an instrument called the Geneva Lens Measure and is procurable as such all over the world. It consists of two peripheral fixed points, and one central point, which is attached to a hand on a watch dial. Originally the two fixed points were 2 cm. apart. These have been altered so that they are now 7 mm. apart. This can be done by the local watchmaker or jeweler. The reason for the alteration is that a flat surface is preferable for application of the stimulus, and it is difficult to find an area of the body that is flat for this distance (2 cm.). Also, too many nerve endings may be stimulated if the points are separated too widely; this may cause summation of impulses. However, readings are approximately the same with either measurement.

A relatively flat, nonfleshy portion of the body is chosen for this test. To insure uniformity in the survey, we have used the proximal phalanx of the

thumb held horizontally with the distal phalanx bent at right angles to it. The sensometer is rested on the phalanx, and the number that it registers of its own weight is kept in mind. Then the instrument is pressed into the skin until the pain becomes unbearable, and the number corresponding to this sensation is read. As one presses the two fixed points harder into the skin, the central point moves an equal distance, and this is measured on the dial. The difference between the figure read before and after pressure on the skin is taken as the patient's pain sensitivity. This instrument is standardized the same way all over the world. It can be tested before using by putting it on a perfectly flat surface; the watch hand will then read zero. In many months of constant use there has been no need for repair of the instrument.

### *DISTRIBUTION OF SUBJECTS*

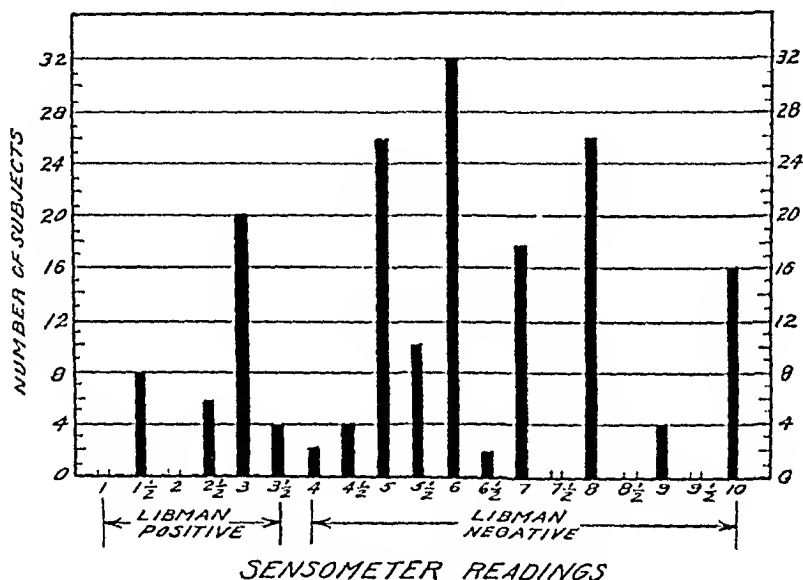


Fig. 2.

### FINDINGS

A total of 178 patients and colleagues were tested repeatedly with the sensometer according to the method described. Each patient was also tested by the Libman method for comparison. The distribution of the results is indicated in Fig. 2. Hypersensitive patients represent about 22 per cent of the total tested, which agrees with the Libman figure of 30 per cent. Except for several minor variations, the Libman test agrees rather well with this study. The majority of the cases that were Libman positive (hypersensitive), ran from 0 to 3.5 units on the sensometer. The Libman negative cases (hyposensitive), with few exceptions, were over 3.5 units on the sensometer. It is in determining the degree of hypersensitivity or hyposensitivity that we feel that this method is superior to the Libman test.

There were 18 patients who deserve separate mention. Eight were positive to the Libman test on one side and negative on the other. Three of these, in spite of being hyposensitive, had had otitis media in their youth, which may account for a tender styloid process. Ten were unusual in one respect, al-

though the agreement between the sensometer readings and the Libman test matched closely. These were neurasthenic individuals, where one would expect a low score and highly positive Libman test, but where the reverse was true. These patients were Libman negative and had a high sensometer rating, but in each the feeling of pain induced by the stimulus lasted from twenty to thirty minutes to several days. Other neurasthenic individuals had low sensometer readings and highly positive Libman tests, but here also the sensation was prolonged. Normally, the feeling of pain caused by the pressure stimulus in either test lasted at most only two to three minutes.

One might postulate a theory from these facts as to the exaggeration of symptoms in certain neurasthenic individuals. There must be constant reinforcement of the stimulus in a neurasthenic individual, perhaps something in the nature of recurring afterimages. This may be caused by lack of certain substances in the body. Wilder<sup>7</sup> has recently restated a well-known fact that pellagrins cannot stand much pain; they feel this pain, however slight, for a long time, its disappearance coming only after adequate nicotinic acid therapy. This suggestive lead is given for research in the pain sensation of some neurasthenic individuals.

Eight patients were hypothyroid individuals. The basal metabolic rates ranged from minus 25 per cent to minus 9 per cent. The sensometer readings were high, some of the highest being in this class. The Libman test agreed very accurately with these readings. Whether these readings were actually high, or whether they were due to a slower perception and slower reaction time, merits separate investigation.

#### SUMMARY

1. A method of estimating the individual's susceptibility to pain is described.
2. This method is compared to the Libman test and a fairly close correlation is reached.
3. Pain sensitiveness in neurasthenic and hypothyroid patients is briefly analyzed.

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## A TUBE FOR THE CENTRIFUGATION OF SIXTEEN SPECIMENS IN A FOUR-PLACE CENTRIFUGE\*

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**I**N LABORATORIES in small hospitals serving large normal populations, the so-called "clinical centrifuge" is adequate for all ordinary purposes, but it may become the "bottle neck" in laboratory procedure in unusual circumstances, prolonging the day's work far beyond the time required by the other operations in the methods, and sometimes resulting in loss of unstable constituents. In a recent survey of vitamin C levels in the plasma of children in an orphanage, the "10 mm. centrifuge tube" shown in Fig. 1 eliminated this difficulty.

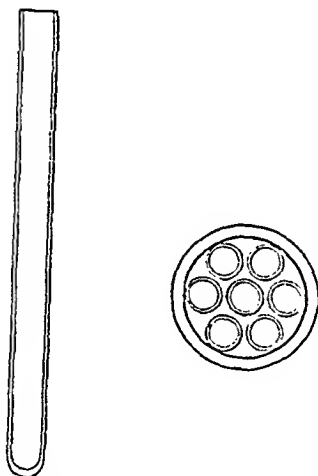


Fig. 1.—A 10 mm. centrifuge tube (left) and the arrangement of seven tubes in a 50 ml. brass centrifuge cup (right).

The International clinical centrifuge accommodates a four-place head having two 15 ml. and two 50 ml. brass cups, which can be purchased separately if the machine is not already equipped with it. One 10 mm. centrifuge tube can be placed in each 15 ml. cup, and seven 10 mm. tubes in each 50 ml. cup. Thus sixteen specimens may be centrifuged at one time. (If flat rubber pads are not at hand for the bottoms of the 50 ml. cups, they can be made by cutting a section from the middle of a No. 6 rubber stopper.)

Although the 10 mm. centrifuge tube was designed for use with the clinical centrifuge having a four-place head, it offers several advantages when used with the larger centrifuge and the eight-place head. Fourteen tubes containing specimens can be balanced in one operation instead of in seven. The depth

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of the supernatant fluid makes it easier to draw off plasma without disturbing the cells. Exposure of the specimen to air is less, whether stoppered or unstoppered. The tube accommodates 4 ml., which is sufficient volume of specimen for many determinations for which blood is taken. It is sufficiently cheap and durable to be used for collection of the specimen, and the volume of specimen is easily estimated (approximately 1 ml. per inch).

The tubing selected for these centrifuge tubes must be between 9.5 and 10.0 mm., *preferably between 9.6 and 9.8 mm. in outside diameter.* The larger limit is the maximum which will permit the tubes to be easily placed in the 50 ml. cups, and the smaller limit insures room enough for inserting the tip of a transfer pipette or a 1 ml. graduated pipette. One end is sealed off as shown, and the tube is cut to a length of 110 mm. and fire-polished. (120 mm. is permissible for use with the larger centrifuge heads.) The end should be smoothly rounded and thick for mechanical strength. Tapered ends similar to those of the ordinary 15 ml. centrifuge tube were tried, but this offered no advantage and was even detrimental in operations in which the precipitate was to be resuspended, such as the washing of cells.

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## THE LAKING OF GLYCERINATED RED BLOOD CELLS BY PHYSIOLOGIC SALT SOLUTION\*

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PERFORMING the Wassermann reaction with high titer amboceptor serum, containing 50 per cent glycerin as a preservative, presents no complications. However, with an amboceptor serum of low titer we encountered a complication which was due to the contained glycerin. After the red blood corpuscles had remained in contact with the relatively large amount of amboceptor serum (necessitated by its low titer) for an hour or longer, they were subject to immediate lysis when added to 0.85 per cent salt solution.

Inquiry revealed that this phenomenon was due to the slow diffusion of glycerin into the red blood corpuscles. The osmotic pressure developed upon introduction into the glycerol-free normal salt solution caused sudden lysis of cells. This degree of osmotic change occurs if the amount of amboceptor necessary to sensitize a given volume of cells results in addition of such an amount of glycerin that its final concentration in the cell suspensions reaches 2 per cent or more.

The glycerinated cells could be brought back to normal stability by washing in hypertonic salt solution or by addition of normal salt solution very slowly in divided amounts.

It is suggested that low titer amboceptor be preserved by some means other than glycerin, or that cells subjected to a large amount of glycerin be suitably washed before use.

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## A RAPID METHOD OF DEHYDRATING AND CLEARING IRON-HEMATOXYLIN STAINED FECAL SMEARS\*

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THE preparation of wet-fixed stained smears for the identification of intestinal protozoa is a time-consuming procedure and is, therefore, considered by many impractical for clinical laboratories. Any modification which shortens the methods used should be of practical interest.

I have found that a preparation called cellosolve (ethylene glycol monoethyl ether) can be used for dehydration and clearing instead of alcohol and xylol, and thereby a considerable amount of time can be saved. Instead of the eight changes usually used for the dehydration and clearing with alcohol and xylol, only a single immersion of ten minutes in cellosolve is required.

The use of cellosolve also has the advantage of being less expensive. A dehydration-clearing set-up of alcohol and xylol (eight dishes) costs about \$1.30, while the cost of a dish of cellosolve for the same purpose is less than 20 cents. Furthermore, the cellosolve can be used longer and for a greater number of smears before losing its efficacy.

By using a short method of staining, as suggested by Johnson,<sup>1</sup> the whole procedure of fixing, staining, dehydration, and clearing can be accomplished in about an hour.

The timing of the various steps in the method now used at the United States Naval Medical School is as follows:

|   |              |
|---|--------------|
| Fix in Schaudinn's solution (glacial acetic acid to strength of 5 per cent added) | 5 minutes.   |
| Alcohol, 95 per cent, with iodine to a port wine color                            | 3 minutes.   |
| Alcohol, 70 per cent  | 3 minutes.   |
| Rinse in tap water.   |              |
| Ferrie ammonium sulfate, 4 per cent   | 15 minutes.  |
| Rinse in tap water.   |              |
| Hematoxylin, aqueous solution, 0.5 per cent                                       | 10 minutes.  |
| Ferrie ammonium sulfate, 0.25 per cent  | 12 minutes.† |
| Wash in running water   | 1 minute.    |
| Cellosolve  | 10 minutes.  |
| Mount in balsam.  |              |

The foregoing method has now been used for a year and has been found entirely satisfactory in the routine work of identifying the intestinal protozoa. As far as can be determined at this time the stains are as permanent as those dehydrated and cleared in alcohol and xylol.

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\*From the United States Naval Medical School, Washington, D. C.

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†Varies somewhat with each batch of hematoxylin. The time may be determined by taking slides through at periods of eleven, twelve, and thirteen minutes, and noting the best result.



# AN IMPROVED METHOD FOR THE ESTIMATION OF QUANTITATIVE AND QUALITATIVE PLATELET FACTORS\*

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IN 1936 we described a method for the quantitative and qualitative estimations of platelets in their own plasma.<sup>1</sup> During the past five years we have shortened and simplified this method until we now believe that it may be easily and simply done in any laboratory.

## METHOD

No anticoagulants are used since such substances mask the extent to which platelets may be broken down in the original blood sample and so interfere with the correct judgment of results. This test requires 1 c.c. of blood, withdrawn from a vein into an iced, oiled syringe, and introduced into a paraffined tube imbedded in ice. We have found that the graduated 1 c.c. tubes used for sedimentation time are excellent for this purpose. These tubes are paraffined with a mixture of two-thirds paraffin and one-third mineral oil. The tubes are then packed in ice in a large centrifuge cup and centrifuged for one and one-half minutes at 5,000 r.p.m..

After one and one-half minutes centrifuging the platelet count is made as follows: A few grains of heparin are placed on a watch crystal which has been thoroughly chilled, and resting in ice. A sample from the center of the centrifuged plasma is drawn up into a capillary tube, and several drops are mixed with the heparin. Following thorough mixing, a drop of this mixture is placed in a chilled counting chamber, covered with a chilled cover glass, and placed in a refrigerator or under ice for fifteen minutes to allow for settling. The platelets in 80 small squares are then counted, and the results are multiplied by 50 to obtain the number of platelets in a cubic millimeter (the platelets in the 80 small squares represent  $\frac{1}{50}$  c.mm.). The platelet counts obtained by the method described must be considered as plasma platelet counts and should be further corrected for whole blood. This is obtained by the hematocrit reading obtained later. The plasma platelet count multiplied by the plasma percentage gives the platelet count for the whole blood.

At the same time that the plasma is placed on the watch glass for the platelet count, the remainder of the plasma left in the capillary tube is used to estimate the coagulation time of the plasma. This is done by breaking off pieces of the tube, as in the method of Rudolph, until a thread of plasma has formed. This represents the coagulation time of the platelet containing plasma,

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and is designated plasma A. As soon as the capillary tube of plasma is withdrawn from the original paraffined tube containing the blood sample, the latter is again placed in ice and centrifuged for fifteen minutes. At the end of this time another sample of the centrifuged plasma, which is now platelet free, is taken into a capillary pipette and the coagulation time is obtained as before. This plasma is designated plasma B. The hematocrit reading may now be made for calculation of the total platelets in the blood sample.

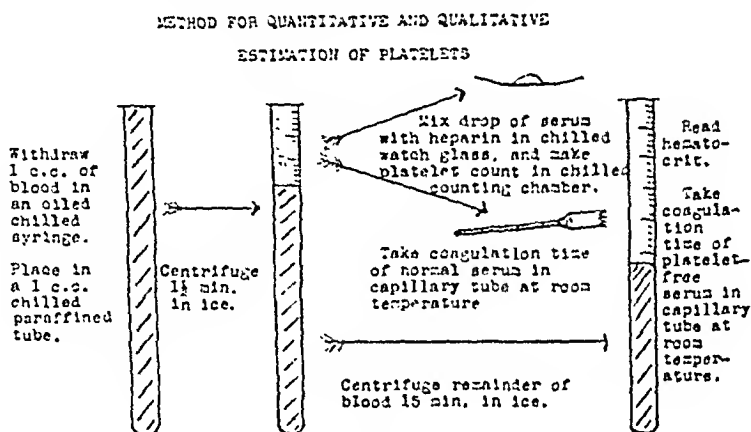


Fig. 1.

The principal changes from our original method reported in 1936 are the elimination of all time-consuming processes dealing with plasma fractions which we do not believe are clinically necessary. Also in shortening the centrifuging of the plasma for fifteen minutes instead of one and one-half hours as formerly used, we have reduced the time so that the entire estimations may be done in twenty minutes. It must be understood that values for plasma B depend on the length of time taken in centrifuging. The longer the plasma is centrifuged, the more platelets will be thrown down, and conversely, the longer will be the coagulation time of this plasma. Fifteen minutes was taken as the time for this centrifuging because it is the shortest time in which all microscopically visible platelets can be centrifuged from the plasma. It would be well before undertaking this test for each worker to test his individual centrifuge by observing if there were any platelets in the plasma after fifteen minutes of centrifuging.

With this procedure we have for consideration a plasma platelet count, the coagulation time of natural plasma with its full number of platelets, and the coagulation time of platelet-free plasma. It must be emphasized that if any difficulty is encountered in obtaining the blood so that there is the slightest addition of tissue fluid or cerebrospinal fluid, the results are unreliable. This can be immediately detected by the platelet count. It will be lower than anticipated, and the platelets will show clumping and variation in size. Any platelet count of less than 100,000 should be viewed with suspicion and repeated. If the procedure is carried out under these conditions, one of two things will occur. First, all the coagulation times will be abnormally short, or plasma B may even coagulate in the centrifuge tube. This should be suspected if plasma A coagulates in less than four minutes. Secondly, in the opposite extreme all

coagulation times may be abnormally long, and plasma B may never coagulate because coagulation has occurred and the supernatant fluid is serum rather than plasma. This is noted especially if cerebrospinal fluid is allowed to get into the sample. This difficulty would probably not occur in obtaining blood from adults, but in pediatric work in newborn and small infants blood is frequently withdrawn from the longitudinal sinus, where cerebrospinal fluid is very easily drawn in with the blood. It is very important, therefore, to know if there have been such additions to the blood. This method of checking is far from perfect, but when anticoagulants are used, no check at all is possible.

#### RESULTS

Normal adults show a plasma platelet count varying between 100,000 and 400,000 per c.mm. The coagulation time for the normal plasma (plasma A) is between four and eight minutes, and for platelet-free plasma (plasma B) between six and ten minutes.

The first deviation from these standards is shown in individuals with hemorrhagic symptoms, but with a normal or higher platelet count. We have found this in the following conditions:

1. Hemophilia. This condition will show values for plasma A and plasma B of from ten to twenty times the normal value. Following transfusion, plasma A will drop to long normal values, but plasma B will still remain long, even during periods that are hemorrhage free. In one instance in a child, aged 1 year, in which the maternal uncle has hemophilia, the platelets were 360,000 with a coagulation time of twenty-four minutes for plasma A and twenty-seven minutes for plasma B. This boy has had some petechial hemorrhages and large ecchymotic areas after small bruises, but no gross hemorrhages have occurred. We feel that the diagnosis of hemophilia is justified in this instance.

2. Pathology of the liver and gall bladder, or bleeding with jaundice. This can be divided into two groups:

a. In which a deficiency of prothrombin also occurs. In these instances both plasma A and plasma B will be somewhat prolonged in proportion to the level of prothrombin. Our ratio has been as follows:

|                          |              |             |
|--------------------------|--------------|-------------|
| Prothrombin 30 per cent, | plasma A 10, | plasma B 11 |
| Prothrombin 40 per cent, | plasma A 9,  | plasma B 11 |
| Prothrombin 50 per cent, | plasma A 8,  | plasma B 10 |
| Prothrombin 60 per cent, | plasma A 6,  | plasma B 7  |
| Prothrombin 70 per cent, | plasma A 6,  | plasma B 6  |

b. In which the prothrombin is of normal value. In several instances we have found an increase in both plasma A and plasma B values with relatively normal prothrombin. For example, in a carcinoma of the gall bladder region, the coagulation time for plasma A was twelve minutes, and that for plasma B was fourteen minutes; the prothrombin value was 75 per cent.

3. A very small group, which except in one instance was characterized by intestinal bleeding, is that in which the platelets are normal or even higher than normal with slightly increased values for plasma A but considerable increase for plasma B. The prothrombin was normal in all instances. With the one exception of an endocarditis with petechial hemorrhages, the remaining four in this group had intestinal bleeding. Three were considered to have

ulcerative colitis. The fourth was a boy whom we have followed for several years, whose case has been diagnosed as Henoch's purpura. In one instance when he was seen immediately after a very severe bleeding from the bowel, his blood showed 325,000 platelets, his plasma A had a coagulation time of nine minutes, and his plasma B eighteen minutes. Since all these patients had normal platelet counts, the delayed coagulation time would seem necessarily due to an increased platelet resistance.

The second and by far the larger group deviating from normal is that in which the platelets are less than normal. We consider this to be the case when the platelets are less than 100,000 by this method.

1. This group includes those with anemias, leucemias, and infections that affect the blood stream. These are all characterized by a thrombocytopenia, proportional increase of coagulation time in both plasmas A and B, and a low hematocrit. We felt that one should never make a diagnosis of primary thrombocytopenia with a low hematocrit. It is almost certain to be of a secondary type. Examples of these are aplastic anemia: Platelets 24,000; plasma A coagulation time nine minutes; plasma B, fifteen minutes; hematocrit 10 per cent; marked hemorrhage. Leucemia: Platelets 124,000, plasma A coagulation time seven minutes; plasma B, eight minutes; hematocrit 40 per cent; no bleeding. Same child when platelets were 23,000; plasma A coagulation time eight minutes; plasma B thirteen minutes; hematocrit 25 per cent; marked hemorrhages. Infected mastoid (*streptococcus*): platelets 40,000, plasma A coagulation time eight minutes; plasma B, fifteen minutes; hematocrit 30 per cent; ecchymosis and petechiæ. After transfusion the platelets were 180,000, plasma A coagulation time was five minutes, plasma B was seven minutes, and hematocrit was 40 per cent.

The findings in these cases of thrombocytopenia suggest that in this group we are dealing with platelets which, though reduced in number, are fairly normal in character. They undergo relatively little disintegration during the process of centrifuging them out of the plasma, and the coagulation time of plasma A is prolonged in most cases, proportionally to the reduction in total platelet count.

2. Thrombocytopenia, with increased coagulation time of plasmas A and B, but with these practically equal in time. The majority of these individuals have had petechiæ and hemorrhages in different parts of the body over a long period of time. A ten-year-old girl had petechial hemorrhages, and bleeding from the mucous membranes for five years: platelets 5,000; plasma A coagulation time eighteen minutes; plasma B, eighteen minutes. A 13-year-old boy had similar symptoms and bleeding over a period of three years, with several transfusions: platelets 4,000; plasma A coagulation time, twenty minutes; plasma B, twenty-two minutes. Following transfusions with increases in the platelet count, plasma A will shorten to almost normal, while plasma B will remain at double the normal coagulation time.

In this group we are dealing with longer standing hemorrhagic disturbances, resembling the syndrome described by Werlhof. Here the platelets do not show a normal resistance but are broken down during centrifugation, yielding a platelet-free plasma which coagulates as rapidly as the normal plasma (plasma A).

3. Thromboeytopenia, with long normal coagulation time, and only occasional hemorrhagic symptoms. Persons of this group have occasional petechiae and small areas of ecchymosis, with some very slight bleeding of the mucous membranes. Their platelets vary from 5,000 to 50,000; their plasma A coagulation time is from four to ten minutes, and their plasma B, from nine to eleven minutes. Their hematocrit, prothrombin, and vitamin C blood content are all normal. We have called such instances "congenital thromboeytopenia," and described a family in such a state in 1936.<sup>2</sup> Since that time we have found twelve more such patients, but have been unable to trace any family connection as in the first family. It will be noted that while their normal plasma coagulates normally, their platelet-free plasma is a little longer than normal. This would indicate that their platelets are very active and that coagulation time is delayed only because of a failure in gross number. Such individuals do not have fatal hemorrhages because their platelets disintegrate more rapidly than do normal platelets. However, some factors might occur that would reduce or lessen this ability to disintegrate and cause a profuse hemorrhage. As long as no exceptional stress or strain is put on persons with this condition, they are able to have normal blood-clotting functions, but in the presence of an acute infection or debilitating condition, the increased power of disintegration of the platelets is lost and a profuse hemorrhage may occur.

One of the most interesting findings to us in this study is the fact that the gross quantity of the platelets in the blood does not seem to make a proportional difference in the coagulation ability of the blood or plasma. Platelet counts of 100,000 to 400,000 will all show approximately the same coagulation values. Even if the platelet value is reduced below 100,000 there is not a great deal of change until the number is decreased below 30,000. We have found individuals with platelet counts of 5,000 and only slightly increased coagulation values. On the other extreme we have hemophilias with platelet values of 500,000, with practically no blood coagulation at all. We have come to pay very little attention to the gross quantitative platelet value, but use it simply as a check on our technique. As stated before, the admission of even the smallest quantity of tissue fluid or cerebrospinal fluid to the blood will decrease the platelet number and give faulty coagulation values. A platelet value of less than 100,000 should be thoroughly rechecked. We believe, therefore, that the most important function of the platelets is not their number or quantity, but their activity or disintegration ability. This can be demonstrated by the coagulation of the plasma fractions as described.

#### SUMMARY

A clinical method for the estimation of platelets quantitatively and qualitatively in their own plasma, without the use of anticoagulants, is described.

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# CHEMICAL

## PEPTONE IN PROTEIN HYDROLYSATES AND AMINO ACID MIXTURES\*

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### INTRODUCTION

**H**YDROLYTIC reagents split protein materials into molecular units of various size. Such degradation products have been classified, supposedly in the order of their molecular weight, as protein, metaprotein, proteose, peptone, subpeptone, and amino acids. Classification is based in part on solubility behavior and reactions which certain hydrolysates display toward reagents.

The peptone fraction of hydrolysates has been the subject of much discussion. Products designated as "peptone" are widely used as a medium for bacterial growth, in jejunal feeding, and in some supposedly therapeutic diets. But even completely hydrolyzed proteins have been decried for parenteral use because of possible "peptone" shock. However, the factor responsible for the production of shock has never been fully established. Two classes of materials have been incriminated: peptone itself, and by-products resulting from the hydrolysis. It has been the common observation that Witte's peptone produces shock<sup>1</sup> or some phase of the shock phenomenon.<sup>2, 3</sup> Thus Henriques and Andersen<sup>4</sup> observed no shock from the parenteral injection of enzymatically hydrolyzed meat protein, yet the injection of Witte's peptone resulted in the death of his animals. Likewise, Milles and Seed<sup>5</sup> gave repeated injections of Difco's bacto-peptone to dogs and were unable to detect any toxic effects. It is thus evident that some peptones produce shock and others do not. It is quite possible that the method used to prepare peptone products is largely responsible for their shock-producing quality, and that "peptone" itself need not necessarily be involved.

It is difficult for the chemist to define what is meant by peptone. The term is a vague one, and though it has been commonly used for years, it does not seem to denote a chemical entity. The several proposals which have been made for its quantitative estimation involve its precipitation from aqueous solution. The earlier procedures utilized phosphotungstic acid as the precipitant, but these methods are now recognized as unreliable due to the partial precipitation of certain basic amino acids which are usually present in the solution. The use of bromine<sup>6</sup> as a precipitant for peptone was discarded at an early date when it was demonstrated that such treatment resulted in the loss of nitrogen. Gubareff and Sergejewa<sup>7</sup> utilized the biuret reaction for estimating peptone after the solution had been heated to coagulate unhydrolyzed protein.

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Such a procedure is unsatisfactory, since it will include all substances not coagulated by heat that give a positive biuret reaction. Schjerning<sup>9</sup> outlined a procedure utilizing tannic acid as the precipitant for the peptone fraction. Bigelow and Cook<sup>9</sup> improved his early technique and claimed that if a correction for creatinine (precipitated by tannic acid) was made, good results could be obtained. They found that a low temperature and a definite concentration of tannic acid and salt were necessary for maximum precipitation of peptone nitrogen. After a careful study of the factors affecting the precipitation of peptone by tannic acid, Wasteneys and Borsook<sup>10</sup> outlined a method for its estimation in enzymic digests of protein under carefully controlled conditions. This is the only available and dependable method for the determination of peptone, and in the experiments below we have followed their outlined procedure.

#### EXPERIMENTAL

An acid and an enzymic digest of casein, a mixture of crystalline amino acids, and Difco's bacto-peptone were studied for their peptone content.<sup>10</sup> The enzymic digest was prepared in a manner previously described.<sup>11</sup> The acid digest was prepared by boiling casein in 25 per cent sulfuric acid and then carefully neutralizing the sulfuric acid with barium hydroxide. The filtrate was decolorized and dried to a fine powder in an atmospheric spray dryer. The mixture of crystalline amino acids contained twenty of the twenty-three usually accepted amino acids in the amounts used by Shohl and Blackfan.<sup>12</sup> Hydroxyglutamic acid, thyroxine, and diiodotyrosin were omitted. Difco's bacto-peptone, as purchased, was the fourth substance.

None of the four substances contained protein or proteose, since they gave no precipitate with trichloroacetic acid or when the solution was saturated with sodium sulfate. The solution was prepared for the peptone determinations by dissolving 10.0 Gm. of the dry powder in 100 c.c. of 22 per cent sodium sulfate solution, adjusting the pH to 7.0 by the addition of a few drops of 60 per cent sodium hydroxide, and diluting to a volume of 200 c.c. with the sodium sulfate solution. To 25 c.c. aliquots were added an equal volume of 2.21 N sodium hydroxide and 125 c.c. of 20 per cent tannic acid dissolved in 0.1 N sulfuric acid containing 20 per cent sodium sulfate. The mixture was thoroughly shaken and kept at 20° C. for four hours. The solution was then filtered and nitrogen determinations were made on the filtrate. All determinations were run in triplicate by the Kjeldahl-Gunning method, and suitable correction made for the nitrogen content of the reagents. The difference between the initial nitrogen content of the original solution and the nitrogen content of the filtrate is expressed as the percentage of peptone nitrogen precipitated.

The results obtained are given in Table I. The apparent peptone content of the enzymic hydrolysate was 26.9 per cent, since this amount of the total nitrogen was precipitated by the tannic acid. The acid hydrolysate gave a figure closely approximating this, i.e., 22.73 per cent. Most surprisingly, the mixture of crystalline amino acids showed an apparent peptone content of 21.36 per cent. These average findings are all so closely similar that they raise the question as to whether all three preparations contain the same amount of peptone, or whether there is any peptone present at all.

It is evident that there is no peptone in a mixture of pure crystalline amino acids. Also, it is generally believed that prolonged boiling with strong acid reduces complex nitrogenous molecules almost entirely to the state of amino acids. At most, some dipeptide linkages may be present. We are, therefore, left with the finding that a mixture known to consist solely of amino acids, and a second mixture known to consist predominantly of such groups, but with possibly some dipeptide linkages, have essentially the same apparent peptone content of 21 to 23 per cent. The conclusion is obviously untenable. The observed differences, if they mean anything at all, do not represent anything in regard to peptone, and at most may represent differences in the amino acid content of the different substances employed.

TABLE I

PRECIPITATION OF NITROGEN FROM PROTEIN HYDROLYSATES AND AMINO ACID MIXTURE

| SUBSTANCE                             | TOTAL NITROGEN               |                      |                        | NITROGEN<br>PRECIPITATED (%) |
|---------------------------------------|------------------------------|----------------------|------------------------|------------------------------|
|                                       | IN ORIGINAL<br>ALIQUOT (MG.) | IN FILTRATE<br>(MG.) | NITROGEN<br>PPT. (MG.) |                              |
| Enzymatically<br>hydrolyzed<br>casein | 150                          | 111                  | 39                     | 26.0                         |
|                                       | 150                          | 108                  | 42                     | 28.0                         |
|                                       | 155                          | 110                  | 45                     | 29.0                         |
|                                       | 155                          | 109                  | 46                     | 29.7                         |
|                                       | 150                          | 115                  | 35                     | 23.3                         |
|                                       | 147                          | 111                  | 36                     | 24.5                         |
|                                       | 147                          | 111                  | 36                     | 24.5                         |
|                                       | 153                          | 104                  | 49                     | 32.0                         |
|                                       | 153                          | 114                  | 39                     | 25.5                         |
|                                       | 153                          | 112                  | 41                     | 26.8                         |
|                                       | 153                          | 114                  | 39                     | 25.5                         |
|                                       | 151                          | 111                  | 40                     | 26.5                         |
|                                       | 151                          | 108                  | 43                     | 28.5                         |
| Average                               | —                            | —                    | —                      | 26.90                        |
| Acid hydro-<br>lyzed casein           | 157                          | 124                  | 33                     | 21.0                         |
|                                       | 160                          | 121                  | 39                     | 24.4                         |
|                                       | 159                          | 120                  | 39                     | 24.5                         |
|                                       | 157                          | 123                  | 34                     | 21.7                         |
|                                       | 157                          | 121                  | 36                     | 22.9                         |
|                                       | 157                          | 125                  | 32                     | 20.4                         |
|                                       | 157                          | 123                  | 34                     | 21.7                         |
|                                       | 157                          | 120                  | 37                     | 23.6                         |
|                                       | 157                          | 124                  | 33                     | 21.0                         |
|                                       | 157                          | 116                  | 41                     | 26.1                         |
| Average                               | —                            | —                    | —                      | 22.73                        |
| Mixture<br>crystalline<br>amino acids | 134                          | 105                  | 29                     | 21.6                         |
|                                       | 139                          | 106                  | 33                     | 23.7                         |
|                                       | 136                          | 107                  | 29                     | 21.3                         |
|                                       | 133                          | 105                  | 28                     | 22.1                         |
|                                       | 133                          | 102                  | 31                     | 23.3                         |
|                                       | 132                          | 104                  | 28                     | 21.2                         |
|                                       | 132                          | 106                  | 26                     | 19.7                         |
|                                       | 132                          | 107                  | 25                     | 18.9                         |
|                                       | 132                          | 105                  | 27                     | 20.5                         |
| Average                               | —                            | —                    | —                      | 21.36                        |
| Bactopeptone                          | 192                          | 50                   | 142                    | 73.96                        |
|                                       | 184                          | 47                   | 137                    | 74.46                        |
| Average                               | —                            | —                    | —                      | 74.21                        |

## DISCUSSION

The report demonstrates the difficulty of arriving at any conclusion on the peptone content of the various materials. A mixture of crystalline amino acids, an acid hydrolysate of casein, and an enzymic digest of casein have been shown to have an apparent peptone content of 21, 23, and 27 per cent, respectively.



The differences in the apparent peptone content of the three widely different substances were not large and can be accounted for solely on the basis of the variability of the method itself. It thus seems impossible to determine what meaning may be attached to the finding that 74 per cent of the nitrogen of a preparation reportedly containing peptone is precipitated by tannic acid.

On the other hand, the precipitation of 74 per cent of the nitrogen of bacto-peptone indicates something. This larger amount of precipitated nitrogen cannot logically be explained on a basis of differing amino acid composition. It probably represents, in part, the presence of some larger molecular units. It seems quite possible, however, that it may not all be representative of such larger units. Nor would it seem advisable to use the figure 21.4 obtained for the mixture of crystalline amino acids as kind of blank for other approximations of peptone, since it can be interpreted from our findings that the amount of nitrogen precipitated by tannic acid depends to some extent on the percentage composition of the particular amino acid mixture used. The mixture of Shohl and Blackfan<sup>12</sup> may or may not approximately represent the composition of the digests, even though their mixture was designed to simulate the composition of casein. The acid digest which we employed contained no tryptophan, and certain portions of other amino acids may have been lost by adsorption on the precipitated barium sulfate. The enzymic digest, although probably containing most of the casein nitrogen, contained in addition a certain amount of nitrogen from the pancreas. We are thus left in doubt as to the significance of the concept of peptone.

#### SUMMARY

Precipitation with tannic acid is the only currently employed method for the estimation of peptone. The procedure results in the precipitation of 21 per cent of the nitrogen of a mixture of crystalline amino acids, 23 per cent of the nitrogen of an acid hydrolyzed protein, 27 per cent of the nitrogen of an enzymic digest of protein, and 74 per cent of the nitrogen of bacto-peptone. The method cannot be regarded as at all definitive for peptone.

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# SIMPLIFIED CALCULATION AND NOMOGRAM OF THE VAN SLYKE UREA CLEARANCE TEST\*

J. E. HEESTERMAN AND S. TILLEMA  
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**D**URING the last decades there has been an increasing interest in the functional capacities of diseased organs. This has given rise to various functional tests. One of the most important of these has been the Van Slyke urea clearance test for renal function. This test has almost generally been accepted as most reliable and has become a routine test in many hospitals. Van Slyke's formula is:

$$\frac{U}{B} \sqrt{C}$$

where  $U$  = urea content of urine,  $B$  = same of blood,  $C$  = urine output in one minute.  $C$  is calculated by dividing the output for one hour,  $H$ , by 60. The result is expressed in per cents of 54, this being the average value in healthy people. The actual calculation, therefore, is:

$$\frac{U}{B} \sqrt{\frac{H}{60}} \times \frac{100}{54}.$$

To reduce the daily loss of time in calculating this formula we used the transposition:

$$\begin{aligned} \frac{U}{B} \sqrt{\frac{H}{60}} \times \frac{100}{54} &= \frac{U}{B} \sqrt{H} \times \frac{100}{54 \sqrt{60}} = \frac{U}{B} \sqrt{H} \times 0.2391 \\ &= \frac{\sqrt{H}}{B} \times 0.2391 U = \frac{\sqrt{H}}{B} \times 0.24 U. \end{aligned}$$

Substituting 0.24 for 0.2391 gives an error of only 0.4 per cent, negligible in routine work. This can be read on a slide rule in a few seconds. However, since medical men in general are not acquainted with the use of a slide rule, we have drawn up a nomogram by transposing the formula to read thus:

$$\frac{U}{B} \times 0.2391 \sqrt{H}.$$

To read the nomogram the figure representing the urea content of the urine and the figure representing the blood urea are connected by a straight line. (A

\*From the Paramaribo Medical School, Dutch Guiana.  
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black thread is most suitable.) The point of intersection of this line and the U/B line is connected with the figure representing the urine output in one hour. The point of intersection of this second line and the scale gives the product of the formula in per cent.

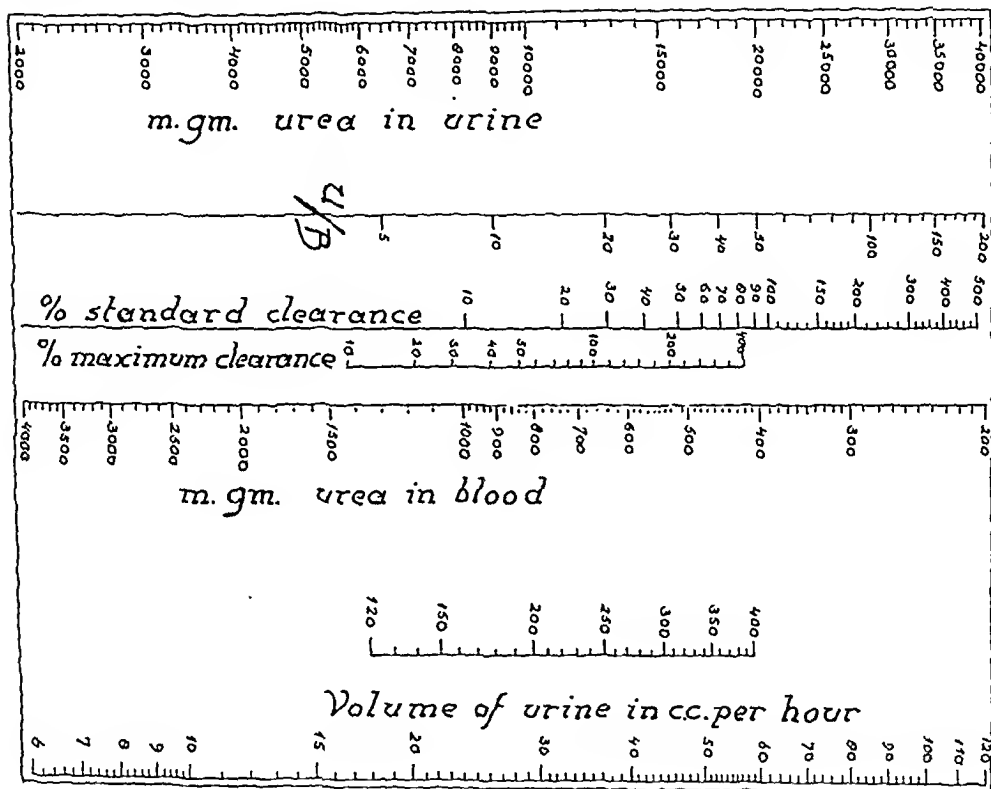


Fig. 1.

A scale for the maximum clearance has been worked out on the same principle. For instance:

$$U = 20,000, B = 1,000, \text{urine per hour } 70 \text{ c.c.}$$

A line from 20,000 to 1,000 crosses U/B at 20. Connect 20 with 70 (cubic centimeters per hour). This line intersects the standard clearance scale at 40; that is, the standard clearance is 40 per cent.

In our medical ward the nomogram has been in use for nearly a year and has proved to be efficient and very simple.

# A NOTE ON THE USE OF HENGAR SELENIZED GRANULES IN THE DIGESTION PROCEDURE FOR THE MICROGASOMETRIC DETERMINATION OF NITROGEN\*

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THE use of potassium persulfate as catalyst and oxidizing agent in the digestion mixture as recommended by Wong,<sup>1</sup> and adapted by Peters and Van Slyke,<sup>2</sup> fails to give consistent results. The destruction of the excess persulfate by the repeated addition of water to the digest cannot be effected uniformly. As a result the oxygen liberated by the reaction of the hypobromite and the residual persulfate in the gas chamber of the Van Slyke-Neill apparatus gives high values. The excess persulfate can be completely destroyed by the addition of oxalic acid, but this is a complicating and time-consuming procedure.

The use of a single Hengar selenized granule in 2 c.c. of a mixture consisting of three volumes of sulfuric acid (sp. gr. 1.84) and one volume of phosphoric acid (sp. gr. 1.71) insures complete digestion in ten to fifteen minutes. Not only is the oxidation catalyzed by the selenium, but bumping is eliminated by the porous granule. This digest is neutralized and treated with hypobromite in the manometric apparatus, as outlined by Peters and Van Slyke,<sup>3</sup> with results which check in duplicate, as indicated in Table I.

TABLE I\*  
NITROGEN CONTENT OF PLASMA

| SAMPLE | VOLUME TAKEN | MG. NITROGEN PER C.C. |
|--------|--------------|-----------------------|
| 1      | 0.1 c.c.     | 11.88, 11.80          |
| 2      | 0.1 c.c.     | 12.06, 12.22          |
| 3      | 0.1 c.c.     | 12.28, 12.10          |
| 4      | 0.1 c.c.     | 12.03, 12.08          |
| 5      | 0.1 c.c.     | 11.58, 11.51          |

\*The results given here are duplicate values on consecutive samples taken in routine experiments.

By this procedure quantities of nitrogen from 0.5 to 1.5 mg. can be determined with an expected accuracy of one-half of 1 per cent. The blank corrections are consistently low.

## SUMMARY

A modified procedure is presented for the preparation of the digest in the microgasometric determination of nitrogen.

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3. Loc. cit., page 357.

Hengar selenized granules may be obtained from the Hengar Company, Philadelphia, Pa.

\*From the Surgical Hunterian Laboratory, Department of Surgery, the Johns Hopkins School of Medicine, Baltimore.

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## A MODIFICATION OF THE PUCHER-SHERMAN-VICKERY METHOD FOR THE DETERMINATION OF CITRIC ACID\*

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THE concentration of citric acid in blood or serum has been a subject of much interest since Sjöström<sup>1</sup> found that this concentration was increased in cases of injuries of the parenchyma of the liver. According to the experience of this laboratory, the test is one of the best laboratory methods for the differentiation of catarrhal jaundice (acute hepatitis) and icterus due to obstruction of the bile ducts. The test does not seem to be so sensitive as the hippuric acid test, though it is perhaps more specific than the latter.

Sjöström and several later investigators used Thunberg's method. In this method the citric acid decolorizes the dye methylene blue by means of an enzyme, citric acid dehydrogenase, from cucumber seeds. The concentration is calculated from the time needed for a certain amount of the serum to decolorize a certain amount of the dye. The method is rather time-consuming, and the decolorization times are not easy to establish with accuracy.

The chemical citric acid method by Pucher, Sherman, and Vickery<sup>2</sup> (the penta-bromacetone method) thus represented a definite advantage. In this method the citric acid is turned to pentabromoacetone, giving a yellow-brown color with sodium sulfide proportional to the amount of acid. The method has been much used in Sweden and other Scandinavian laboratories, and has proved to be satisfactory.

This method has, however, one drawback. The color developed is rather unstable and it fades in such short time that it can scarcely be used for colorimetry. For this reason, Pucher, Sherman, and Vickery mixed the final solution with pyridine to a concentration of about 40 per cent in order to stabilize the color. Using the method in routine clinical work, we have found that the pyridines from different factories give very different color strengths to the final solution of the same amount of acid. Even when the pyridine was distilled at a low temperature, its stabilizing ability soon grew weaker, and the color strength decreased. Only one of the tested pyridines did not change in this way. Moreover, the extinction coefficient of the pyridine mixture was not always directly proportional to the citric acid content of the solution. Sometimes a small amount of citric acid, 20  $\gamma$  or less, did not give a higher extinction than the blank. Finally, the extinction coefficients plotted against the citric acid amounts did not form a straight line, as can be seen from the values of the

\*From the Medico-Chemical Laboratory of the Medical Department, Karolinska Sjukhuset (The Carolinian Hospital), Stockholm.  
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extinction coefficients divided by the amount of acid in Table I. On the other hand, the color once developed is very stable in pyridine solution (Table II).

TABLE I

Extinction coefficient (E) of varying concentrations of citric acid, and the proportion between this coefficient and the concentration (E/mg.). In the last column correction has been made for the amount of acid which did not give any visible reaction.

| γ CITRIC ACID | GLYCEROL TWICE DISTILLED |       | PYRIDINE    |       |            |       |
|---------------|--------------------------|-------|-------------|-------|------------|-------|
|               |                          |       | REDISTILLED |       | PURISSIMUM |       |
|               | E                        | E/mg. | E           | E/mg. | E          | E/mg. |
| 0             | 0                        |       | 0           |       | (-0.030)   |       |
| 50            | 0.070                    | 1.40  | 0.040       | 0.80  | 0.040      | 1.40  |
| 100           | 0.145                    | 1.45  | 0.095       | 0.95  | 0.110      | 1.40  |
| 200           | 0.290                    | 1.45  | 0.180       | 0.90  | 0.250      | 1.40  |
| 300           | Too dark                 |       | 0.360       | 1.20  | 0.390      | 1.40  |

In order to find a better color-stabilizing substance a great number of solvents have been tried as substitutes for the pyridine. The best one proved to be pure glycerol (glycerinum bidestillatum). When this solvent is used, the color is much stronger than that obtained with the best pyridine, and the extinction coefficient is directly proportional to the concentration of the acid (Table I). On the other hand, the color was not absolutely stable, and started slowly fading after two hours (Table II). This, however, is of no practical consequence. Glycerol of commercial quality may be used, but it gives a somewhat weaker color (Table II).

TABLE II

Extinction coefficient of the solution from 100 γ citric acid made up to 10 ml. read for different periods after the preparation.

| MINUTES | GLYCEROL        | PYRIDINE   |             |
|---------|-----------------|------------|-------------|
|         | TWICE DISTILLED | COMMERCIAL | REDISTILLED |
| 0       | 0.150           | 0.130      | 0.095       |
| 60      | 0.150           | 0.130      | 0.095       |
| 120     | 0.150           | 0.130      | 0.095       |
| 240     | 0.145           | 0.120      | 0.095       |
| 300     | 0.130           | 0.110      | 0.095       |
| 390     | 0.120           | 0.100      | 0.095       |

Among the other substances tested, acetone and saturated sodium chloride solution had some stabilizing effect, but they were much inferior to glycerol.

In the experiments of which the results are given in the tables the penta-bromoacetone was extracted from its solution in petroleum ether three times, with 3, 2, and 1 ml., respectively, of sodium sulfide solution, according to Pucher, Sherman, and Vickery. The sulfide extracts were transferred to 10 ml. bottles containing 4 ml. of the stabilizing solvent. The solution was made up to volume with the same solvent and read with the light filter S:47 (which was found more convenient than S:43, the filter used by Pucher, Sherman, and Vickery).

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## AN AID IN THE DITHIZONE METHOD: A MECHANICAL SHAKER FOR SEPARATORY FUNNELS\*

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ONE of the most frequent operations in the determination of lead by the mixed-color dithizone method, as used in this laboratory,<sup>1, 2</sup> is the shaking of the separatory funnels for the purpose of extracting various components from one phase or the other of the mixture. Although each shaking period is only a minute in length, this operation is repeated at least four additional times during the course of each determination. Experience has shown that in making thirty analyses during a day, more than three hours are consumed in shaking when this is done by hand. The tendency is to decrease either the speed or the intensity of shaking when the operator becomes tired.

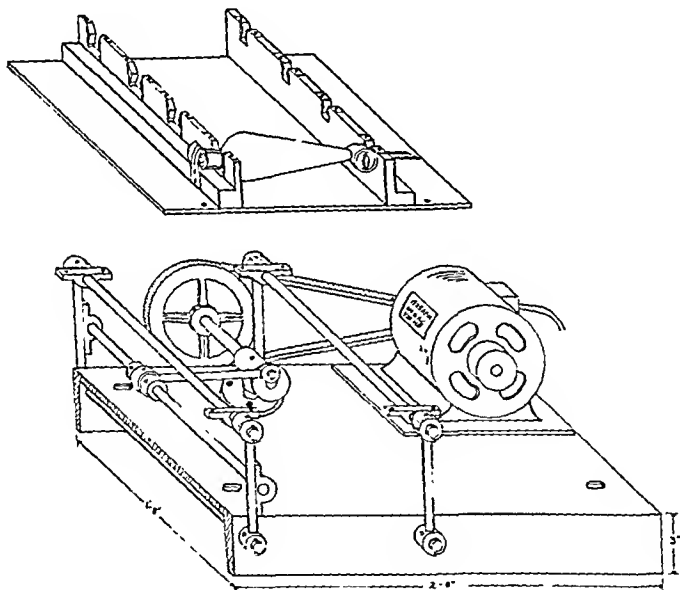


Fig. 1.

In order to expedite the analyses and to obtain uniformly vigorous shaking a mechanical aid was devised (Fig. 1). This consists essentially of a maple rack to hold four 250 ml. Squibb type pyrex glass separatory funnels, this rack being given a reciprocating motion by means of an eccentric driven by a  $\frac{1}{20}$  H.P. electric motor 1,725 r.p.m. The figure indicates the component parts of the device, with the rack removed to show details of the driving mechanism. The

\*From the Division of Industrial Hygiene, National Institute of Health, United States Public Health Service, Federal Security Agency.  
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connecting rod crankpin is offset  $\frac{9}{16}$  inch from center of eccentric, and the center-to-center length is  $7\frac{1}{4}$  inches for the connecting rod and  $6\frac{5}{8}$  inches for the four rocker arms. The heavy section of channel iron is bolted to a large box (18 by 28 by 20 inches high) filled with sand and mounted on No. 14 rubber stoppers to absorb the vibration.

The most difficult task is to hold the funnels securely, since they vary not only in length but also in the outside diameter of neck and stem as well as in length of neck and stopper. Of the several arrangements tried, the one described is the most satisfactory. Phosphor-bronze spring stock was bent to form clips as shown, so that after pushing the funnel down past the middle of the slot there is no tendency for the funnel to loosen up during shaking. The distance between the two slotted boards is slightly greater than the longest funnel in use. The spring holding the stopper in place has sufficient tension to prevent the stopper from coming out and allows for any differences in length of neck. By this arrangement no metal comes in contact with either funnel opening, thus avoiding contamination.

The funnels can be rapidly inserted and removed from the holders using only one hand. With pulleys 1 inch and 6 inches in diameter about 350 double oscillations are produced per minute. With an electric timing switch added, the minute occupied in shaking the four funnels can be used to carry out other operations. Two of these shakers, constructed in this laboratory, have given satisfactory service for over two years.

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# MEDICAL ILLUSTRATION

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## WATER COLOR DRAWINGS FOR SCREEN PROJECTION

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HERETOFORE a transparent base has not been used extensively for hand-drawn pictures in color and tone. Such water color illustrations are made to serve as lantern slides primarily, but in the past color in flat washes or lines was used. Color also has been applied over photographs in tone, thereby producing the tonal effect. By experimenting over a period of six months, a method has been found for making a picture that is smooth enough to be projected on a sizable screen for lecture purposes. Of course, since color photography has taken such an important place in the illustrative field, the scope of the subject material for hand-colored lantern slides has been reduced. However, the hand-made slide still serves those items which cannot be photographed as clearly as they can be drawn, such as peritoneoscopic, bronchoscopic, and cystoscopic subjects.

When a transparent medium first was considered, several different materials presented themselves as surfaces upon which to work, for example, glass, cellophane, cellulose, film and other available transparent materials. I tried putting a clear varnish, similar to the one used by artists, on cellophane to keep it from wrinkling, but it became streaked when dry. It was then put on glass, but the same result was obtained. Ammonium hydroxide washed upon blue-tinted roentgenographic film and allowed to dry worked fairly well so far as shading of the different parts was concerned, but it changed some of the colors, for instance, blue became green.

About this time an article written by Dr. M. Musehat on "A New Technique in Medical Illustration" appeared in this JOURNAL. The article dealt with colored illustrations for lantern slides and was accompanied by a black-and-white print of a colored drawing by the author which did not seem to be smooth enough for projection. I was able to obtain some of the material he used, and on examination found it to be similar to other cellulose acetate and cellulose nitrate film, except that it had a ground surface on one side that was excellent to draw upon in pencil. When water colors were applied, however, they became slightly gray and could not be worked to a smooth finish. In short, the film was difficult to handle. Numerous experiments were made to determine which solution and which material would work together to produce this smooth finish. A bit of liquid glue in a small amount of water works well when applied to the film, but after this solution dries it cannot be softened sufficiently to scratch off the high lights without spoiling the film underneath. However, it is an aid in putting on a smooth tone and should not be discarded entirely.

The best method to use in making a smooth lantern slide is to prepare a solution of one part of gum arabic crystals to eight or ten parts of water. This may be thinned if desired. Paint the whole surface of a piece of blue-tinted roentgenographic film from which the gelatin surface has been removed by soaking it for a few minutes in warm water, and allowing it to dry. The water colors can then be used in the ordinary way. When the colors are dry and you are ready to scratch off the high lights, dampen the end of an etching tool which has been sharpened to a fine point and carefully scratch off the paint and the solution. The film itself will show through the solution and give the appearance of the highest high light. Insert the film upon which your drawing has been placed between two lantern slide cover glasses and bind with lantern slide binding tape. If you wish, you may make a mat of black opaque paper for the drawing and put it between the two cover glasses before they are bound.

Another medium upon which such colored drawings can be made is the cleared but unexposed and undeveloped lantern slide plate. For example, take a new lantern slide plate from the pack and place it in a photographic fixing bath. This bath clears the plate and leaves a piece of glass with a transparent film base. Such a base supplies the "tooth" to hold the water color pigments which should be applied in thin washes. It is the custom of medical illustrators to scratch or to engrave high lights out of their drawing base or surface rather than to apply white or light pigments with a brush. Because the lantern slide is a transparency, it is essential that the high lights be engraved out rather than built up. In applying the water colors to the surface as described here, the surface becomes damp and, therefore, unsuitable for engraving out the high lights until it is thoroughly dry. Once it is dry, it answers the purpose satisfactorily.

Many water-color pigments have an opaque base and are entirely unsuitable for the coloring of lantern slides and similar transparencies. For instance, the true earth pigments, such as yellow ochre and raw sienna, are particularly opaque and therefore unsuitable. All whites are opaque in varying degrees. For this reason, truly transparent colors should be used. These colors include some of the aniline dyes, which are invariably synthetic derivatives. Most of the dyes used in staining microscopic slides are of the transparent variety, but all of these do not lend themselves to the coloring of transparencies owing to their lack of permanence. The degree of permanence can be ascertained from a majority of the reputable manufacturers of colors. A fair degree of permanence is essential because the rays of light passing through a lantern slide are so great or intense at the point where the slide is placed in the machine that fugitive pigments will change rapidly. This change is caused by the light and heat intensity produced by the average projection bulb. The purchaser can do little more than rely on the word of the pigment manufacturer as to the permanence of his product. However, the transparency of a pigment can be tested by observing a small amount of it under a microscope with transmitted light. By this method the tiny particles of a pigment which appears to be of a definite color by reflected light become black or gray by transmitted



Tubo-ovarian abscess.



Tubercular lesion of the colon and abdominal wall.

*These are typical examples of the method of illustration described in the text.*



light. Genuine transparent pigments retain their color when studied by transmitted light. The artist must, therefore, choose a palette composed of transparent colors if he is to color lantern slides. Even if one opaque pigment gets mixed in with the rest of the palette a lack of transparency and brillianee will result. In art this is termed *muddiness* or *muddy color*.

A set of transparent water colors may be bought from most art and photographic supply houses in book or liquid form. These serve well for the purpose.

As a rule, it is best to dampen the working surface before the colors are applied. A peneil sketch on paper may be placed beneath the transparent material to serve as a guide in applying the color.

It must be remembered that any flaw, particularly a dark one, will show up when the slide is projected. Therefore, it is advisable to make the picture with great care. I use a stamp collector's magnifying glass, which has a swivel neck and a stand, through which to work. It aids materially in making such illustrations.

# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILBUFFE, M.D., ABSTRACT EDITOR

**TUBERCLE BACILLI, Detection of, by Fluorescence Microscopy, Bogen, E. Am. Rev. Tuberc. 44: 267, 1941.**

Sputum or other material, preferably after concentration, is thinly smeared on a glass slide. The slides may then be stained with any acid-fast staining technique, except that the carbolfuchsin is replaced by auramine. The staining solution is made by placing 1 Gm. of pure auramine powder in a flask, gradually adding 1,000 c.c. of distilled water, shaking strongly until it is completely dissolved, and then adding 50 c.c. of pure, liquefied phenol, and again shaking. A temporary clouding later settles out, leaving a clear yellow solution which keeps for a long time in the dark. Hagemann recommends staining with auramine for fifteen minutes at room temperature, and decolorizing with acid alcohol without counter-staining.

The following procedure, after Herrmann, has been used at the Olive View Sanatorium:

1. Fix with heat.
2. Flood with auramine solution, and steam for five minutes.
3. Wash in running water.
4. Decolorize in 70 per cent alcohol, containing 3 per cent hydrochloric acid, until the preparation is colorless.
5. Wash in running water.
6. Dip in a 0.1 per cent aqueous solution of potassium permanganate (this solution remains usable for about a week after turning brown).
7. Wash in running water.
8. Dip in Löffler's alkaline methylene blue solution.
9. Wash in running water.
10. Dry and examine with the fluorescence microscope.

Fluorescence microscopy is a new method for detecting tubercle bacilli. Its theoretical basis suggests advantages over the present microscopic procedures, both in speed and delicacy. An intense violet or ultraviolet source of radiation is required. The bacilli are stained with the fluorescent dye, auramine, and examined with an ordinary microscope equipped with special light filters. Practical experience confirms its predicted value. In 1,000 duplicate smears stained in parallel by the Ziehl-Neelsen and fluorescence methods, there were over 20 per cent more positive tests by the newer technique. In 250 parallel cultural and fluorescence microscopic examinations, the specificity of the latter was confirmed, although the cultural methods still gave considerably more positive findings.

**JAUNDICE, Significance of Rise of Non Protein Nitrogen in, Meyer, K. A., Popper, H., and Steigmann, F. J. A. M. A. 117: 847, 1941.**

The nonprotein nitrogen of the blood is increased in patients with both medical and surgical jaundice of severe involvement. The increase is more pronounced in cases in which there is a fatal outcome.

The determination of the nonprotein nitrogen is of diagnostic, and especially prognostic, value in such cases, since it points to an aggravation of a parenchymatous jaundice and to the appearance of a secondary liver damage in cases of obstructive jaundice. Increase of nonprotein nitrogen in a case of surgical jaundice suggests an early operative intervention.

The increased nonprotein nitrogen is to some extent explained on the basis of a reduction of glomerular filtration, but to a greater extent is due to increased reabsorption of urea in the kidney tubules.

An increased breakdown of protein as a cause of the increased nonprotein nitrogen is not evident from the authors' experiments.

**BACTERIOLOGICAL TECHNIC:** Improved Method of Obtaining Bacteriological Specimens From Wounds Treated by the Closed Plaster Method, DeWaal, H. L. Brit. M. J., Aug. 23, p. 268, 1941.

After the wound has been cleaned, sterile thick absorbent thread is laid backward and forward across its surface, starting at the center and passing to the periphery. The free end of the thread lies away from the wound. The whole of the wound surface is now covered with sterile vaseline gauze. The free end of the thread is passed through a "one-piece" vulcanite cylinder with a flat base and with the sharp edges smoothed off. (The vulcanite should be of good quality and able to resist autoclaving.) A groove is cut in the flat base of the cylinder in which the thread lies. This allows for the subsequent easy withdrawal of the thread. A sterile rubber stopper is inserted into the upper opening of the cylinder. While the plaster is being applied, an assistant steadies the cylinder at the edge of the vaselined gauze (about 1.5 cm. from the wound margin). In taking specimens the procedure adopted is as follows:

The rubber stopper is first removed, and with a swab slightly moistened in antiseptic the inside of the cylinder is cleaned and then dried with another swab. That portion of the thread which had been within the cylinder and had come in contact with the antiseptic is now withdrawn, cut off, and discarded. A further piece of the thread is pulled out (about 1.5 cm.), snapped off, and placed in a sterile test tube. This portion of the thread has been exposed to air, and should be examined as a control. The next few centimeters are similarly collected, and give a representative sample of the wound flora.

Depending on the length of the thread originally laid on the wound, five or more specimens may be taken at varying intervals. The last portion of the thread comes from the center of the wound, the part likely to heal last. After each specimen is taken, a fresh sterile rubber stopper is inserted in the mouth of the cylinder.

**LYMPHOCYTOSIS, Infectious, Smith, C. H.** Am. J. Dis. Child. 62: 231, 1941.

Two types of lymphocytic blood reactions are described to which the term infectious lymphocytosis has been applied.

In one type, which occurs infrequently, unexpected hyperleucocytosis with absolute and relative lymphocytosis appears for a short period and is not accompanied by any recognizable symptoms or physical signs. In the two cases of this type reported in this paper the maximum white blood cell counts were 44,300 and 98,000 cells per cubic millimeter, respectively.

The second type is frequently encountered in pediatric practice and includes cases in which after an infection of the upper respiratory tract low-grade fever continues to be present for prolonged periods. Associated symptoms include anorexia, pallor, fatigability, and para-umbilical pain. The blood in such cases shows a lymphocytic preponderance instead of the expected neutrophilic response and moderate leucocytosis. This symptom complex occurs most characteristically in infants and in young children.

Examination of bone marrow in three cases showed an increase in lymphocytes as the only abnormality.

These two types are classified as acute and chronic infectious lymphocytosis, respectively, and are differentiated clinically, hematologically, and serologically from infectious mononucleosis, leucemia, and miscellaneous infections associated with lymphocytosis.

It is pointed out that many lymphocytic blood responses in children whose titer of heterophilic antibody is normal belong in the class of infectious lymphocytosis rather than infectious mononucleosis.

Acute and chronic infectious lymphocytosis may represent separate entities, although there is some evidence that they may be related.

The etiologic agent of each type is probably not represented by the bacterial flora obtained from the nasopharynx, but probably is an as yet undetermined virus related to infection of the upper respiratory tract.

The prognosis in both types of infectious lymphocytosis is favorable. Treatment of the chronic type is symptomatic and should be directed toward relieving nasopharyngeal infection.

**CHICKENPOX, The Blood Picture in, Holbrook, A. A. Arch. Int. Med. 68: 294, 1941.**

This study was made during an epidemic of chickenpox in Milwaukee County in the fall of 1939. The 56 patients and 15 controls were all quarantined in their homes.

The following significant tendencies are noted in the patients:

1. The total white blood cell count falls during the end of the incubation period, reaches the lowest level on the second and third days of the rash, and then rises to its former level.
2. During the leucopenia the percentage of polymorphonuclear cells falls, and the percentage of lymphocytes rises, as lymphoblasts and prolymphocytes come into prominence. Among the latter, the mitotic figures, which are occasionally seen, attest further to the strong stimulus to lymphocytic activity which apparently accompanies chickenpox.
3. An increase in plasma cells is also noteworthy early in the eruption stage, their portion of the total differential count sometimes reaching 6 to 8 per cent.
4. In some instances, several of the foregoing features may appear before the rash and thus may be of diagnostic value.

The studies on the control group show the following tendencies:

1. In the aggregate, the various plotted curves for total white blood cell counts and their fractions tend toward a horizontal line.
2. In certain individual cases, a fall in the percentage of polymorphonuclear cells and a rise in the percentage of lymphocytes, with prolymphocytes increasing, occur at about the time the eruption is expected.

**VITAMIN C and Serum Protein Levels in Wound Disruption, Hartzell, J. B., Winfield, J. M., and Irvin, J. L. J. A. M. A. 116: 669, 1941.**

The authors would emphasize Mason's statement that "the urge to heal is almost as great as the urge to live." Wound healing is affected by many factors, and the nutritional condition of a patient is a most important one. Either to maintain life or to repair tissue there must be a supply of proteins, fats, carbohydrates, minerals, water, and vitamins. Periods of inanition or faulty nutrition are likely to deplete the carbohydrates and fat stores and to lower serum protein and vitamin levels. The authors almost universally supply carbohydrate and salts by routine intravenous administrations, but, generally speaking, if a transfusion is given, it is because the hemoglobin and the red blood cell count are low rather than because the serum protein is low, and the vitamins are forgotten entirely. It would seem logical that, if normal healing is to be expected, the ascorbic acid and serum protein levels of the blood plasma should be brought to normal concentration and kept there.

**MENINGOCOCCI AND GONOCOCCI, Simple Method for the Isolation and Identification of, Boisvert, P. L., and Fousek, M. D. Am. J. Dis. Child. 61: 710, 1941.**

Twenty-six strains of meningococci and 8 strains of gonococci were isolated in the pediatric laboratory during a three-year period; the carbon dioxide jar of Thompson was used for the cultures. The organisms grew out well in twenty-four hours on blood agar plates and were recovered from material from all infected patients on the first culture. It was found that the simple addition of the differentiating carbohydrates to the blood agar afforded a satisfactory method for further identification of the organisms by fermentation tests.

*Media.*—A fresh beef heart infusion with 1 per cent neopeptone was the basic medium used. The blood agar plates used for the isolation of the organisms were made by adding 10 c.c. of defibrinated rabbit's blood to a flask containing 200 c.c. of melted infusion agar. The blood and agar were mixed, and the mixture was poured into sterile Petri dishes and allowed to solidify. The contents of one flask made about fifteen blood agar plates.

For the fermentation tests blood agar plates containing dextrose, maltose, and levulose were used. Five-tenths cubic centimeter of defibrinated rabbit's blood was added to a tube containing 10 c.c. of melted infusion agar. The contents of the tube were mixed and



poured into a sterile Petri dish containing 2 c.c. of 10 per cent dextrose. An even distribution of blood agar and carbohydrate was obtained by gently tilting the dish back and forth while the contents were still liquid. Blood agar plates with maltose and levulose were made in the same manner.

*Carbon Dioxide Jar.*—The apparatus consisted of a round, flat-bottomed 2.5 liter pyrex jar, a square cover with corked aperture, and a container for reagents. It differed only in minor details from the apparatus used by Thompson. The container for reagents was fastened to the bottom of the jar with sealing wax. Petrolatum was used to seal the cover to the jar.

Sodium bicarbonate and sulfuric acid were the reagents used. The sodium bicarbonate solution was made by dissolving 84 Gm. of sodium bicarbonate in 1 liter of water. The sulfuric acid solution contained 20 c.c. of concentrated sulfuric acid per 580 c.c. of water. Equal amounts of each reagent were used, and 22.4 c.c. of carbon dioxide were formed from each 1 c.c. of sodium bicarbonate solution. An amount of each solution, equal to 0.45 per cent of the volume of the jar, furnished an atmosphere of about 10 per cent carbon dioxide.

*Isolation of Strains.*—Purulent discharge from the eyes or the genital tract was obtained on a sterile cotton-tipped wooden applicator. It was smeared over a small area near the edge of a blood agar plate and streaked with a platinum loop.

A 0.1 c.c. sample of spinal fluid was placed on a blood agar plate with a sterile pipette and streaked in a similar manner.

Blood cultures were made by adding 5 to 7 c.c. of the patient's blood to 50 c.c. of infusion broth and by mixing 2 c.c. of the citrated blood of the patient with 10 c.c. of infusion agar to form a pour plate.

Material for culture was obtained from the nose by introducing a cotton-tipped wooden applicator into the nostrils until the nasopharyngeal wall was reached; material from the throat was secured by touching both tonsils and the posterior portion of the pharynx. The material from the nose and that from the throat were streaked separately on blood agar plates.

The inoculated media were put into the carbon dioxide jar, the calculated amount of sodium bicarbonate solution was placed in the container for reagents, and the jar was covered. An amount of sulfuric acid equal to that of the sodium bicarbonate solution was added through the hole in the cover by means of a pipette. The hole was closed with a cork, and the jar with its contents was incubated overnight at 37.5° C. Portions of colonies on the plates which resembled meningococci or gonococci were smeared on a glass slide, stained, and examined microscopically. Blood cultures positive for these organisms were examined in a similar manner. If gram-negative diplococci were found, the organisms were further identified by their fermentation reactions.

*Fermentation Tests.*—Three blood agar plates, containing dextrose, maltose, and levulose, respectively, were inoculated and incubated overnight in the carbon dioxide jar. One drop of 2 per cent bromthymol blue was placed on each of the plates in an area where colonies were present. Fermentation of the sugar was indicated by a change in the color of the indicator from dark blue to yellow.

**BLOOD SERUM, Desiccated, Viability of the Spirochetes of Syphilis and Yaws in, Turner, T. B., Bauer, J. H., and Kluth, F. C. Am. J. Med. Sc. 202: 416, 1941.**

*T. pallidum* and probably *T. pertenue* are commonly killed by the process of freezing and desiccation, even when the method is such that the viability of many other bacteria and viruses is retained. Transfusion of desiccated blood serum or plasma is, therefore, probably without risk as regards the transmission of syphilis or yaws, even though the material is obtained from an infected donor.

## REVIEWS

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Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, 201 West Franklin Street, Richmond, Va.

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### Genital Functions and Their Hormonal Regulation\*

INTERNATIONALLY known for his work in endocrinology, Dr. Zondek in this monograph presents and discusses the results of investigations carried on in the Hebrew University and the University Hospital in Jerusalem.

In six main sections are discussed: I. The Occurrence of Some Estrogenic Substances in Nature; II. Experimental and Clinical Investigations of the Percutaneous Use of Estrogenic and Androgenic Hormones; III. The Effect of Prolonged Treatment With High Doses of Estrogenic Hormone; IV. On the Fate of Estrogenic Hormones in the Organism; V. Clinical Investigations Concerning the Cycle of the Female Organism; VI. The Mechanism of Menstruation.

Of interest is the report in the opening section of the demonstration of estrogenic hormone and a flavin derivative (vitamin B<sub>2</sub>) in the water and sandy slime of the Dead Sea.

In Section III Dr. Zondek reports absence of any evidence that prolonged hormone therapy may be, as has been suggested, a factor in carcinogenesis.

Space does not permit any extended résumé, but it can be said that these studies are of great interest and value to the investigator in this field and to the clinician as well because of their clinical aspects.

Well printed and excellently illustrated, this is a valuable contribution. A summary completes each chapter and a bibliography is appended.

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### Essentials of Endocrinology†

AS THE author comments in his preface, the field of endocrinology is marked at the present time by "feverish activity." His book, therefore, will be welcomed by those who wish to have at least a bird's-eye view of what has now become, in many respects, a fundamental science.

In this book Dr. Grollman presents a well-balanced survey of the present status of endocrinology and discusses in seriatim: Endocrine Glands of the Cranial Cavity; The Bronchiogenic Organs; Endocrine Glands of the Abdominal Cavity; Hormones of the Reproductive System; and The Hormones Derived From the Nonendocrine Glands.

A useful reference list of available endocrine products is found on the cover pages, front and back.

Well oriented and organized, well written and illustrated, this is a useful book deserving of wide circulation.

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\*Clinical and Experimental Investigations on the Genital Function and Their Hormonal Regulation. By Bernhard Zondek. Cloth, 264 pages, 59 figures, \$4.50. Williams & Wilkins Co., Baltimore, Md.

†Essentials of Endocrinology. By Arthur Grollman, Ph.D., M.D., Associate Professor of Pharmacology and Experimental Therapeutics, Johns Hopkins University, Medical School. Cloth, 480 pages, 74 illustrations, \$6.00. J. B. Lippincott Co., Philadelphia.

In Memoriam



Dean De Witt Lewis  
1874-1941

Associate Editor  
The Journal of Laboratory and Clinical Medicine  
1926-1941



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## PROGRESS

### SOCIAL REVOLUTION AND THE PHYSICIAN\*

WARREN T. VAUGHAN, M.S., M.D., RICHMOND, VA.

LET us for a moment turn the calendar back eight short years, to the year of our Lord 1933.

This was a year of depression. The banks took a holiday. Even so, we were making progress. The eighteenth amendment was repealed, destroying the problem of racketeers. The Industrial Recovery Act was passed, assuring prosperity. The bonus grab was consummated, distributing billions of dollars, that our country might lift itself by its bootstraps. Chicago was celebrating a Century of Progress. Wiley Post was circling the world in eight days. The Golden Gate Bridge was abuilding and the first streamliner train was entering service. Science and medicine were making strides. Heavy water was being discovered; magic eyes were opening doors. Surgeons were removing entire lungs. The Rockefeller Foundation was spending fifteen million dollars to promote the well-being of mankind.

All was reasonably well with the world.

On January 30 a fiery little fanatic, Adolf Hitler, became Chancellor of Germany. Within the year Mower wrote a book, *The Brown Book of the Hitler Terror*. Armstrong wrote another, *Germany Puts the Clock Back*. The Nobel Peace Award for 1933 was postponed.

Will that year go down as the nineteen hundred thirty-third from the birth of Christ? Or will it gain more lasting place as the year "One" of the New Dark Ages?

There have been glorious epochs in history. A high point was the Age of Pericles, when commerce and industry, art, philosophy, and the science of the

\*Convocation address at the opening of the University of Michigan Medical School, October 4, 1941.

time flourished. We read of the rulers and sages of that age, and call it Golden. We forget that its average persons lived as crudely as the barbarians who later destroyed them. We forget that the glories of ancient Greece were built upon a foundation of slavery.

If I had my choice of any half century in which to live during the last fifty thousand years, I would select the years just elapsed and would request life in a democracy, preferably in the United States. At no other time or place in the history of creation have men, from the wealthiest and the most intelligent to the poorest and the least intelligent, enjoyed comparable comfort and freedom.

True, this period has witnessed disease and crime. Utopia is not yet here, but it seemed almost within reach. At what point on the road of progress did our race stray into the bypath which has led us to the brink of disaster?

During the first World War a new element entered those factors which appeared to be controlling progress. A few recognized this at the time. Many do so now when it is almost too late. This new element is complex. It is a trend, best analyzed in the changes in social psychology since the last war.

In the intervening decades there has been continuous advance in the physical or mechanical aspects of living. Automobiles and ships have improved. Diesel trains, air-conditioned buildings, and other inventions have made living almost too comfortable. Scientific achievement is gaining, not losing, momentum. New discoveries are now heralded daily, and the science and the art of medicine are at a high point of achievement.

In these aspects the world appears to have been traveling onward toward grander things. The downward change has been in our attitude toward life, in our interpretation of the reason for existence, and in our evaluation of those things for which life is worth the living. I might say that in a mechanized world, our philosophy has become mechanistic.

Many factors have played a part. The first, a direct outcome of World War I, was man's unconscious denial of the value of human life. Only by the adoption of such an attitude can soldiers live through a war of destruction without going insane. When one sees dear friends, stalwart young men, blown to fragments, knowing that the next shell may be his own, one forgets the platitudes of the Sunday-school teacher on God's interest in the weakest lamb of the flock. The success of wars depends upon mass training in the psychology of the unimportance of the individual. All of us who were in France absorbed it, whether we were fighting with bayonets or merely closing the eyes of others as they died from violence or disease.

Such a psychological attitude persists after its need has passed.

A second factor which in this country has changed the outlook of the masses was prohibition, which made gentlemen out of drunkards and drunkards of gentlemen; which allowed honest men to deal with crooks; which made connivers of policemen and bars into speakeasies; and which bred contempt of the law in every class, but most regrettably in children, as they watched their parents flout it and laugh.

I find a third conditioning influence in the present trend toward mediocrity in productive effort, especially among the laboring groups. Time was when craftsmen and their apprentices derived as much satisfaction from the making of an artistic copy of a famous statue, painting, saddle, nutcracker, or bookmark as the artist had in his original creation. Today, the trade unions discourage originality. All must be stereotyped. No matter what the degree of one's ability, all must work the same hours and receive the same pay. Creative ambition among craftsmen is discouraged. Once there were cabinet makers. Then there were carpenters. Now there are saw-and-hatchet men who piece together jigsaw puzzles of prefabricated materials.

These three influences, mediocrity, lawlessness, and negation of the importance of the individual, have probably been the most important factors in this country. The trend has been world wide. It is illustrated in the communism of Russia and the corrupt politics of France.

There is a fourth factor which has influenced nations. While Germany has been the leader in this factor, it has been reflected in her allies, Japan, Italy, and the Russia of 1940. It may be summarized in the term national dishonesty. If a government lies, cheats, steals, and murders, the ethics of those living under it must of necessity undergo subtle changes.

We are living in a period of world revolution, of turmoil, in which the socialistic trend, the trend toward mediocrity, the tendency to draw the best down toward the worst is dominant. We have seen civilizations fall. Is today's situation comparable to the softening that caused the downfall of Rome? Are we witnessing a temporary eclipse of civilization?

For myself, the answer is no. But I must qualify this answer.

We are entering a new world order, one which may not make the world better. We are in a phase comparable to the French Revolution. It involves a reshuffling in which the "haves" will become "have nots," but the "have nots" will not become "haves." The outstanding phenomenon of the catastrophe is the destruction of individualism. Individualism was destroyed in the French Revolution, but it returned. It will always return.

How have we managed to get ourselves into this predicament? Have we taken a byway off the path of progress? Or have we reached a previously uncharted area on the main road? Are there more basic conditioning elements than the superficially obvious ones which I have described? I see three such possibilities.

The first is the influence of cosmic forces. The suggestion has been made that those solar convulsions which cause sun spots may extend their influence to things living on earth. It has been shown that periods of social unrest coincide fairly well with phases of increase in sun spots. This applies to the World War, the Russian Revolution, and even to runs on the stock market. When William Petersen is showing how terrestrial weather may influence man physically, mentally, and emotionally, we need not stretch imagination to grant the possibility that cosmic rays or other solar forces might influence our activities. When C. A. Mills tells us that the upward trend in college student stature appears to be undergoing a reversal, and that sexual maturity is occurring at

a later age; when he attributes this to the unseasonable warmth prevailing over the earth since 1929; and when he remarks that the physical recession of mankind during the Dark Ages occurred during centuries of world warmth, we must ponder on the possible influence of cosmic forces on human progress.

We turn from such conjectures, since their acceptance implies a defeatist attitude. But we cannot ignore them if they may be true. If we grant their possibility, then our every effort should be to counteract such deterministic tendencies with whatever means science may make available.

We must consider human factors as well as geastral forces. There are two: the growth of science, and the failing influence of religion.

The Christian religion has been the greatest social force for good that the world has ever known. I say this with full realization of the hypocrisy, injustice, corruption, torture, and brigandage that have been committed in the name of Christ. The Jewish, Buddhist, and Confucian religions have been comparably potent for good, although probably less widely effective. A single discovery nearly 400 years ago blasted the tenets of all theologies. When Copernicus proved that the earth revolves about the sun, our planet could no longer be the center of the universe. It became just a little ball, gyrating in unison with countless other little balls.

Men of science have been forced by their discoveries to become agnostic. They find no proof of individual life hereafter. They have adjusted themselves to the philosophy of contributing greatest good for the greatest number on this earth, and to the knowledge of immortality at least in the germ plasm of one's descendants. Surely we can speak of the religion of science. Members of this cult are today leading a more ethical life than are some so-called pillars of the church, even though the former have no fear of eternal damnation or hope of reward in the hereafter. In this way, the advanced thinkers have solved their problem. The trouble is that, with the popularization of science, nonthinkers have also become convinced that there is no hereafter. The great controlling force of orthodox religion over men with antisocial tendencies has been the fear of hell fire. With this gone, potential criminals are all out for what they can get in this life. Destruction of the concept of immortality, brought about by science, has been a potent factor in increasing crime on this earth.

We are living in days of realism. This applies even in the young. A five-year-old Jewish boy had heard his parents discuss the tragedy of the Jews in Germany. One day, disobeying injunctions, he went to the river bank where, of course, he fell in. His Mother told him that God, angry at his disobedience, had punished him. Next time God might let him drown. To which the lad replied, "I hate God." His reasoning was logical: "Why should God do that to me, when he does nothing to Hitler?"

The deity myth is one of the most remarkable phenomena in history. Anthropomorphic God failed his only Son two thousand years ago. Did we accept this as evidence of nonexistence? No, we made excuses, we explained that he wanted Christ to suffer like man. And this man-made illusion has continued



to govern the world for two millennia, fortunately so, because we were not ready for the next step in human evolution.

It has been said that the one hope for dogmatic religion is the arrival of a new Messiah. He must come from the lowest classes. He must fire the imagination. His religion must be militant. It must offer promise of heaven on earth. Today some see such a Messiah. His name is Adolf Hitler. He is the most evil genius that the forces of creation have ever let loose upon this earth.

What may we expect from science? Scientists have realized that average man must have a tangible religion. To this extent they have regretted the passing of the old. Some have attempted to substitute a religion of ethics based upon science. Their writings usually start convincingly and end in a philosophic hodgepodge which leaves their readers and, I suspect, themselves in a complete fog, proving that the scientist should limit himself to science and leave metaphysics and philosophic dissertations to those better qualified in the field.

One writer, Edwin Conklin, has avoided the pitfalls. In his essay "Does Science Afford a Basis for Ethics?" he formulates the answer more clearly than any other whose writings it has been my pleasure to read. Briefly excerpted, his reasoning is as follows.

"There is evidence that in times long past there were types of human beings, more brutish in body, mind and social relations than the general average of the present race. There is abundant evidence that ethics has undergone evolution no less than intelligence. It seems incredible that reason, emotion, aspiration and ethics should develop out of such simple functions as sensitivity, reflexes, trial and error, and yet these incredible things are actual facts.

"Psychical development begins with the ability to respond differently to stimuli of different quality or degree. Just as the amoeba avoids extremes of hot or cold, all living things grow toward the satisfactory and avoid the unsatisfactory. This is the beginning of psychic life. It is based upon the ability to distinguish between that which is satisfactory and that which is not, and to follow after the one and to avoid the other. This very fact of distinguishing and choosing or following is the basis of wisdom. Freedom comes with the ability to make a choice between two alternatives, and is measured by the extent to which remembered experience influences behavior. Intelligence is the capacity of profiting by remembered experiences.

"The highest level of human development is attained when purpose and freedom joined to social emotion, training and habits, shape behavior not only for personal but also for social satisfaction, since society no less than the individual is seeking satisfaction. When all these things combine, we have what we call ethics or the science of right conduct.

"The process of evolution involves not only the physical body but mental attributes and ethical reactions. Ethical or social reactions can be measured in groups of laboratory animals as easily as physical attributes. Evolution occurs in the body, the mind, and in social relations.

"Among savage tribes ethics or altruism pervades the tribe but does not extend beyond. In the higher social grades altruism reaches farther and takes in more people, until with some persons it includes the whole human race."

Cyril James has defined society as "an organism which we can envision as endowed with a kind of independent life that enables it to continue in existence despite the procession of births and deaths that determines the lives of the men and women who compose it."

We may gather several inferences from these statements. "Survival of the fittest" does not apply only to brute force. The prize fighter is not best fitted to fly a plane, to operate a submarine, or to organize an ideal social group. In evolution the highest men have developed not only physically and mentally but also socially or ethically.

The distinctive attribute of Jesus was that he was from 2,000 to 4,000 years ahead of his time. In the evolution of man, as far as ethics is concerned, he was precocious. There were others such as Moses and the Greek philosophers. With increasing numbers in later centuries, progressively fewer stand as far above their fellow men.

Socially, Christ was a perfect man. Plato was another, of almost comparable stature. His "Republic" is still the ideal to be attained, although it may be another two thousand years before we reach it.

There is another inference that I would draw from Conklin's essay. In the process of evolution among social organizations the democracy is farther advanced than any other form of government. The difficulty with the democracy of today is that, although it has a thousand superior ethical thinkers to one superior thinker of two thousand years ago, the mass of its members is not yet sufficiently evolved. When evolution has proceeded to that stage where all have acquired ideal ethics, then democracy will be perfect. I believe that time will come.

A corollary is that the Germanic tribes of today are not at as high a stage of evolution as the English, the Scandinavian, the American, and those other political communities which have demonstrated their capabilities for self-government. The German people have no genius for this. We blamed Wilhelm in 1914. We blame Hitler now. If there had been no Kaiser or no Hitler there would have been someone else. The trouble is with the mass psychology of the Teutonic race as much as it is with its rulers. The ethos, the code of ethics of the democratic states, is one of "give and take." The Nazi code is simply that of "take."

Our error at the last peace was the assumption that democratic government would be appropriate for men who were insufficiently evolved to handle it. If the democracies win this war a benevolent authoritarian government must be set up for Germany.

I find food for thought in the observation that while the Celts and then the Teutons first settled northern Europe, the Saxons and Angles broke away from the latter, to inhabit the British Isles. These could not tolerate a static life any more than their descendants who colonized America. Those who pushed always farther into the unknown where they could develop new and better social organizations may have been those farthest advanced in evolution. If we accept the cradle of the Aryan race as in Western Asia, and compare the Slavs, the

Tentons, and the Anglo-Saxons, we may conclude that the farther they have gone geographically the better has been their capacity for self-government.

I do not wish to imply that the democracies have a monopoly of the finest minds. There have been superior ethical men in Germany, also in the Slavie nations and elsewhere. Many in Germany have been Jews, but some have been Teutons. The fact remains that when the Mediterranean races were in the flower of civilization, the Germanic tribes were still barbarians. For all of Hitler's vaunting of his personal group of Aryans, and in spite of the many fine minds that have lived in Germany—and escaped from Germany—compared with the most highly evolved groups in the world today, those who rule Germany now are one thousand years behind. They are still in the period of rule by deceit, cunning, and might. Their social ethics has not yet matured.

When Hitler entered Poland he told his people that he had taken a step which would change the history of the world for a thousand years. If he wins, this will be true. Human progress will have to step back one thousand years, and hope that a thousand years hence it will be back where it now is. I say this advisedly. Hitler's policy is one of destruction of the thinkers, the progressives, the ethical. During the two thousand years since Christ we have reached that stage where there is an appreciable, almost a militant, proportion of ethical persons. With their destruction, the rule of the barbarian will be complete, and the clock will have been set back ten centuries.

What will this world be like a thousand years from now? Kirtley Mather tells us that the present supply of oil in the United States will be exhausted in thirty years; the known world's supply, within seventy-five years. We have enough coal to last two thousand to three thousand years. Science can now make gasoline and similar products from coal. But there will be an increasing demand for nonrenewable products, such as coal and metals. Mather estimates that the demand will be doubled within a few decades. There will also be an increase in population. He concludes, "Taking all these things into consideration it would appear that world stores of needed natural resources are adequate to supply a basis for the comfortable existence of every human being who is likely to dwell anywhere on the face of the earth for something like a thousand years."

May I, for the sake of rhetorical effect, stretch my imagination and ask you to stretch yours to visualize a Hitler victory which will set the world back a thousand years; which will result in colossal wastage of nonreplaceable resources as is occurring today in the sinking of ships, the destruction of property, and the wanton explosion of those things which should be used by superior man in his daily life, ending a thousand years from now in the bankruptcy of our resources and the extinction of the human race?

Mather tells us that species like the dinosaurs, which have become extinct, lived an average of half a million years before they vanished. *Homo sapiens* has had only fifty thousand years of existence. Must we let Hitler and his minions rob us of four hundred and fifty thousand years?

Thirty years ago Prussian scientists were boasting racial superiority. Today it is Aryan purity. Then, it was superman. The superman of today is less

common among the Germans than in the more socially integrated democracies. Arrogance, intolerance, and sadism are not his characteristics.

During the era of splendid German scientific achievement, around the turn of the century, they were so positive, so dogmatic, so intolerant of dissension that they convinced not only themselves but many others of their intellectual superiority. After several decades the clay feet commenced to show. I was amused recently by the writings of some leading Germans regarding the causes of hay fever and asthma. Their conclusions of 1900 were as wrong as they could be. Today we know that hay fever is not a bacterial infection, and that asthma is not primarily a neurosis, but if you were to read those forty-year-old essays today and were not acquainted with progress in the intervening period, you would be as convinced as they were of the infallibility of their conclusions.

No, superman will not be defined in terms of the physical or mental prowess of the individual, but rather in his adaptation as a unit in the social structure. Christian kindness, not arrogance, will be his outstanding characteristic. He will not hesitate to fight if necessary, but only for truth and justice. Even Jesus drove the money changers from the temple. I say *Christian* kindness because Christianity is still an ideal religion. Take away the concept of the supernatural which was added after Christ's death. Take away the promise of personal immortality. What have you left? We still have the keystone—the teachings of Christ—a creed of moral and social ethics which has no superior.

We still have the Golden Rule.

The Prussian's conceit almost convinced us that his was the race of supermen. We are startled to discover that with the rationalization of science we ourselves have better claim to this distinction. If this is true, we have a heavy obligation, the task of preserving the higher civilization from destruction by those less advanced races which have assumed a camouflaging veneer of culture and are now using the implements of science, devised for social progress, for destroying society.

Here are you young men and women, at the beginning of your life's career, full of enthusiasm and ambition, and the feeling that all is reasonably well with the world. And here am I, an apostle of semigloom, invited here to wish you Godspeed and success. By way of apology and explanation may I say to you that I have four sons, all headed for careers in medicine. As a father, world problems have given me deep concern. I can only say that the advice I am giving you is the advice that I have given my own sons.

There have been other purveyors of gloom. The man who said "there is scarcely anything around us but ruin and despair" was William Pitt, the date 1800. The Duke of Wellington on the eve of his death in 1851 "thanked God that he could be spared from seeing the consummation of ruin that was gathering around." Disraeli ten years earlier had written, "In industry, commerce and agriculture there is no hope." Lord Shaftesbury said in 1848, "Nothing can save the British Empire from shipwreck." A hundred and forty years ago Thomas Jefferson wrote that dictators will "destroy every trace of civilization in the world and force mankind back into a savage state."

Today it seems truer. It may be because of our proximity or because the social upheaval is more world wide. Undoubtedly the future will be different.

What are you young men and my sons and I and the rest of us to do about it? I can give you only the high points.

First, you must prepare yourselves. Some of you are about to finish your medical education, others are just beginning. There will be a shortage of doctors and you will aid in keeping this at a minimum.

Next, your function will be to help win the war, for I can assure you it is our war, a battle against extinction of the most highly developed groups of the human race. Once you are in it, war is not a horrible affair. It is a thrilling experience. Otherwise it would not have been so popular through the ages. There is a mass psychology which destroys the sense of fear. You may be killed; if so, you will not remember it. You may be maimed, and this is the sad part of war; in the present barbaric struggle this applies equally to noncombatants. The chances are better than even that you will come through unscathed. There will be hardships, possibly heartbreaks, but we who went through the last war know that these are speedily forgotten and that in retrospect, and indeed in the day-to-day experience, fighting is lots of fun.

After we have won the war or, God forbid, lost it, what then? Present trends presage increasing socialization, involving scientists and physicians with the rest of humanity.

May I present three general alternatives? First, you can attempt to change the trend, whatever it may be, in an effort to retain present-day customs. This had best be left to geniuses or fools.

Second, you can "play the game." If individualism is temporarily eclipsed in America, and preventive and therapeutic medicine are to be regimented along with public health administration, enter this new day with enthusiasm and a spirit of cooperation. If medicine must become bureaucratic, do your best to reach the top in the new order, where you can do your part in making state medicine the finest type of medicine. Do this with only one object in view, to uphold the spirit and tradition of the best in our profession, applying it to the new order. In this way will you achieve lasting and self-respecting success.

If, again God forbid, we return to the Dark Ages, your function should be that of preserving the advances made up to now, while awaiting the dawn of a new era. Your lives should then be comparable to those of the monks of the Middle Ages, the monasticism which kept knowledge alive, albeit feebly, until the new day. In short, the third alternative is that of cloistered existence in research and teaching in schools of higher education.

There is a fourth alternative. It has neither the spectacular appeal of the first, the active appeal of the second, nor the self-abnegation of the third. It is the program which will be followed by the rank and file of you, the life that has made medicine a glorious profession, and has made doctors beloved by their people. This consists in aiding the sick, advising the disturbed, encouraging the feeble, comforting the bereaved, building health and happiness for the individual and letting the more ambitious worry about the first three alternatives.

Physicians are, on the whole, more highly evolved ethically than many other groups. Furthermore, the doctor is in less conflict with current politico-social trends than are others. The future will bring fewer basic changes in medicine than in other fields.

Each of us will find his function in one or another of these categories, and each will be doing his part to preserve the human race from deterioration. We are fortunate in that none of the schedules calls for wanton destruction of our fellow men, although this is mandatory for others, if humanity is to persevere. Nevertheless, we should be willing to fight, with arms if necessary, for that which we know to be best for the human race. Today, Hell hereafter disturbs us not at all. But Hell on earth is at our doorstep. Let us all become members of the new religion of science, the religion of moral and intellectual decency; a religion which is so jealous of human progress that it is willing to destroy ruthlessly all deterrents; a religion of ethics, whose decalogue may some day contain no prohibitions—no “Thou shalt nots”—but whose code of human relationships is summarized in the English phrase, “Is it cricket?”

It would seem appropriate in closing to read you a few words from an essay on “The Evolution of the Superman,” written many years ago by Dean Victor C. Vaughan, who was beloved by all who knew him during his forty years’ service with this School.

“Men are mortal but man is immortal. The individual lives are at best but a span, but the race continues. Numberless finites make up the infinite, and yet the soundness of the part determines the perfection of the whole.”

It rests, then, with us as individuals, each to do his part, great or small, in assuring to posterity the continued upward progress which ethical man has striven so laboriously to deserve.

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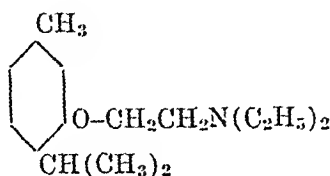
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# CLINICAL AND EXPERIMENTAL

## PHARMACOLOGY OF THYMOXYETHYLDIETHYLAMINE\*

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THYMOXYETHYLDIETHYLAMINE is a phenolic ether with the following chemical structure:



The free base is an oil, insoluble in water. The readily formed hydrochloride dissolves in water, a 3 per cent solution having a pH 3.0. Such solutions are unstable, primarily because of photosensitivity of the compound; however, they may be kept intact for a period of months in sealed ampoules under nitrogen and stored in the dark.

This compound was first described by Anan<sup>1</sup> who called attention to its sympathicolytic properties resembling those of the ergot alkaloids. Asakura<sup>2</sup> showed that it could inhibit the appearance of the adrenalin-induced hyperglycemia in the intact animal. Bovet and Maderni<sup>3</sup> showed that thymoxyethyldiethylamine, together with a number of other phenolic ethers, was capable of producing an ergotoxin-like reversal of the pressor effect of epinephrine on anesthetized dogs; this action they attributed to a paralysis of the sympathetic end plates. Levy and Ditz<sup>4</sup> made a similar observation on thymoxyethyldiethylamine and a number of its substitution compounds, describing the action as similar to that of ergotoxin or yohimbine in its ability to cause a reversal of the usual epinephrine effects.

Okazaki<sup>5, 6</sup> has given a detailed description of the pharmacologic properties of the methyl homologue of this compound, thymoxyethyldimethylamine. He points out that the compound has an effect as potent as that of ergotamine on the isolated rabbit uterus, that it is considerably less toxic than ergotamine (frogs), and that it is capable of causing a reversal of the typical adrenalin pressor effect. According to Staub,<sup>7</sup> this compound is in use in Japan as a therapeutic agent under the name "Tostramin."

Another closely allied substance, the diethylamine ethyl ether of 2-methoxy-6-allyl-phenol, has been described by Eicholtz,<sup>8</sup> and by Schmidt and

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Scholl,<sup>9</sup> under the proprietary name "Gravitol," and has been used clinically as an oxytocic agent in the treatment of uterine inertia, etc.

Fourneau and his co-workers<sup>10-12</sup> pointed out that certain phenolic ethers, particularly thymoxyethyldiethylamine, have the capacity of counteracting the effects of histamine, both *in vitro* and *in vivo*. The typical histamine-induced contractions of isolated smooth muscle could be inhibited by the previous addition of this compound to the bath fluid, and guinea pigs could be protected from the parenteral administration of two lethal doses of histamine by the previous subcutaneous administration of doses of 40 mg. per kilogram of the thymol derivative. Similar doses could also save sensitized guinea pigs from lethal doses of horse serum which produced fatal anaphylactic shock in unprotected animals.<sup>13</sup> This protection was achieved without influencing the sensitizing-desensitizing mechanism, for such animals behaved in the same way as controls when a subsequent precipitating dose of horse serum was administered some days after the administration of the thymoxyethyldiethylamine.

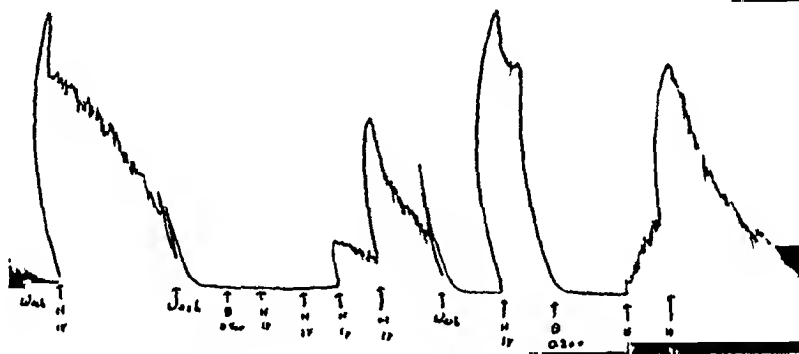


Fig. 1.—Loop of guinea pig jejunum in Webster's solution containing atropine. The normal response to the addition of 1 $\gamma$  of histamine to the bath fluid, followed by a wash and the addition of 0.4 c.c. of a 0.01 per cent solution of thymoxyethyldiethylamine. The normal histamine response is only observed after 1 $\gamma$  of histamine have been added. The preparation is washed, and the response to 1 $\gamma$  of histamine is again obtained. The addition of 0.2 c.c. of thymoxyethyldiethylamine causes an immediate relaxation of the contraction inaugurated by the histamine, and another normal histamine response is evoked after the addition of 2 $\gamma$  to the bath.

More recently, Rosenthal and Minard,<sup>14</sup> and Lambert and Rosenthal,<sup>15</sup> have described thymoxyethyldiethylamine as having a specific protective action against histamine and have used this observation to support their thesis that it is the local liberation of histamine which gives rise to the sensation of cutaneous pain. They have described thymoxyethyldiethylamine as possessing a local anesthetic action of about twice the potency of novocaine, but have failed to mention the intense local reactions resulting from the subcutaneous administration of this substance. We have repeatedly observed local edema, local necrosis, and sloughs following the subcutaneous administration of 1 per cent solutions. Staub has also called attention to this local effect and has pointed out that one drop of a 1 per cent solution will cause persistent congestion and laceration when placed on the rabbit's conjunctiva, and that urticarial papules may be produced when the material is passed through the skin by iontophoresis.

Staub has shown that thymoxyethyldiethylamine is capable of inhibiting the response of the isolated loop of guinea pig jejunum to doses of histamine



which would ordinarily induce maximal contractions. We have also observed this effect and have found that a quantitative relationship exists between the amount of histamine inhibited and the amount of thymoxyethyldiethylamine present. Thus, as illustrated in Fig. 1, doubling the amount of thymoxyethyldiethylamine added to the bath doubles the amount of histamine antagonized. Staub has shown that thymoxyethyldiethylamine not only inhibits the oxytocic effect of histamine but also causes almost complete inhibition of the normal response of acetylcholine and a suppression of the normal response to barium chloride. Rosenthal and Minard, however, claim that the antagonistic action of thymoxyethyldiethylamine for histamine is specific, and that small doses, capable of causing complete suppression of the isolated preparation's response

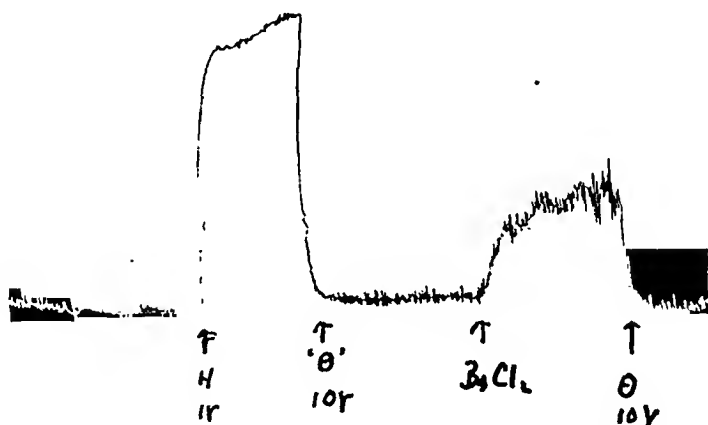


Fig. 2.—Loop of guinea pig jejunum in Webster's solution containing atropine. A maximal response is elicited by the addition of 1% of histamine to the bath. The addition of 0.1 c.c. of a 0.01 per cent solution of thymoxyethyldiethylamine causes immediate relaxation of the muscle. The preparation is washed, and a second contraction is initiated by the addition of 20 mg. of barium chloride. Relaxation occurs immediately when the same quantity of thymoxyethyldiethylamine is added to the bath.

to histamine, have little or no effect on the contractions resulting from the addition of barium chloride, acetylcholine, sodium sulfocyanide, potassium chloride, or tyramine to the bath fluid. Our results do not bear this out, but are in closer agreement with those of Staub. We have found that quantities of the thymol derivative which will cause suppression of histamine contractions of the isolated gut will also render that preparation incapable of responding to barium chloride, and that concentrations which will cause immediate relaxation of smooth muscle after histamine stimulation will have a similar effect on contractions resulting from the administration of barium chloride. This observation is not compatible with any claim for specific antihistamine action. Sufficiently high concentrations of most spasmolytic or narcotic agents will curtail or completely inhibit the responsiveness of smooth muscle to oxytocins. Katz<sup>10</sup> has pointed out the fact that chloral hydrate, urethane, and ether will inhibit the response of smooth muscle to histamine. Downs and one of us (D. R. C.)<sup>11</sup> have shown that dibromoethylene behaves similarly with respect to histamine and the ergot alkaloids, but does not inhibit the contractions in-

itated by the addition of pituitrin. This does not mean that these substances have a specific antihistamine effect, but rather that the muscle fibers are narcotized, or rendered incapable of responding to oxytocic stimuli. We are of the opinion that this is equally true of thymoxyethyldiethylamine.

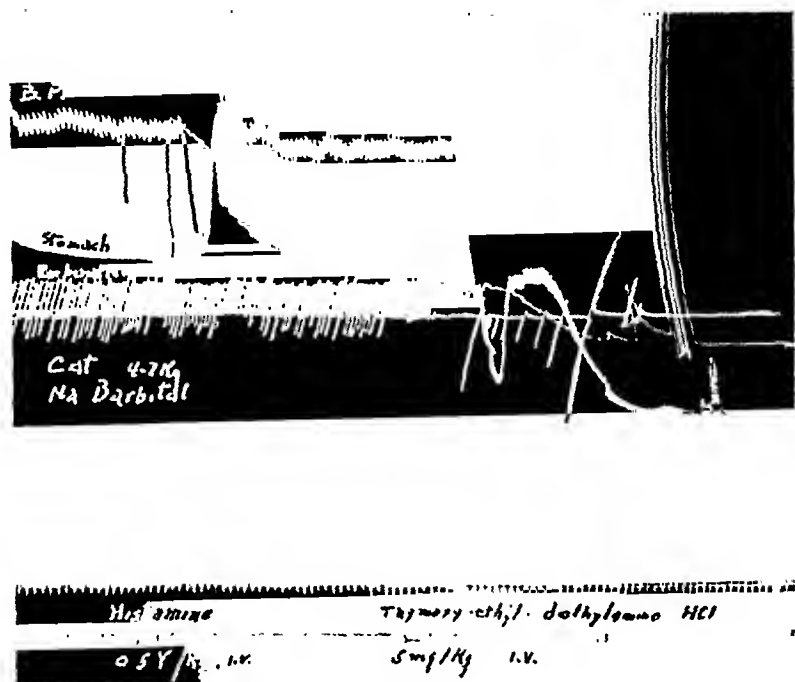


Fig. 3.—Cat under sodium barbital anesthesia. Record of carotid blood pressure, gastric contractions, and respirations. Time in five-second intervals. The fall in the blood pressure and the contraction of the smooth muscle resulting from the administration of 0.5% of histamine per kilogram are first recorded. This is followed by the intravenous administration of thymoxyethyldiethylamine (5 mg. per kilogram) which causes the death of the animal.

The parenteral administration of thymoxyethyldiethylamine to intact animals gives rise to a number of dramatic effects. To begin with, the compound is quite toxic. Staub gives the toxicity in Table I. She points out that toxic

TABLE I

| ANIMAL     | M. L. D. (MG./KG.) |             |
|------------|--------------------|-------------|
|            | SUBCUTANEOUS       | INTRAVENOUS |
| Guinea pig | 200                | 20          |
| Mouse      | 500                | 25          |
| Rabbit     | 200                | 25          |

symptoms occur after the administration of doses as small as 10 per cent of the minimal lethal dose. We have observed fatal outcomes from the administration of doses as small as 4 mg. per kilogram intravenously to cats under sodium barbital anesthesia. The first effect is a slowing of the respiratory rhythm associated with a diminution in the amplitude of the respiratory movements. This is followed immediately by a precipitous fall in the blood pressure which is maintained at the shock level for about one minute. During this time apnea sets in. When the apnea has become complete, the heart fails and the blood pressure falls to zero.

It should be pointed out that the vasomotor system is rendered incapable of responding to potent stimulation during the time the blood pressure is at the shock level, for the administration of epinephrine in doses as large as 0.5 c.c. of a 1:10,000 dilution failed to evoke a significant response.

The intravenous lethal dose was determined for adult male rats, weighing between 350 and 400 Gm. The results, which are shown in Table II, are of the same order as those described by Staub.

TABLE II

| NUMBER OF ANIMALS | DOSE (MG./KG.) | RESULTS   |      |  |
|-------------------|----------------|-----------|------|--|
|                   |                | RECOVERED | DIED | NOTES  |
| 2                 | 3.0            | 2         | 0    | Nil  |
| 2                 | 6.0            | 2         | 0    | Nil  |
| 2                 | 7.5            | 2         | 0    | Nil  |
| 4                 | 9.0            | 4         | 0    | Convulsive movements during and immediately after administration. Rapid recovery |
| 2                 | 12.0           | 2         | 0    | As above   |
| 2                 | 13.5           | 2         | 0    | As above. Marked dyspnea   |
| 2                 | 15.0           | 2         | 0    | As above. Symptoms more marked   |
| 5                 | 16.5           | 3         | 2    | Deaths from 0.5 to 5 minutes after administration                                |
|                   |                |           |      | Dyspnea and convulsions  |
| 10                | 18.0           | 2         | 8    | As above   |
| 3                 | 19.5           | 0         | 3    | As above   |

Subcutaneous toxicity was determined on mice, and here again our figures agree with those obtained by Staub. Doses as small as 25 mg. per kilogram, however, produced marked inflammatory reactions at the sites of injection. When the dose level was increased to 100 mg. per kilogram, the inflammatory reaction was intense; the skin and subcutaneous tissues first became erythematous and edematous, the skin underwent necrosis, and the involved tissues sloughed away. Table III shows the results of the subcutaneous administration of an aqueous solution (1 per cent) of thymoxyethyldiethylamine hydrochloride to mice.

TABLE III

| NUMBER OF ANIMALS | DOSE (MG./KG.) | RESULTS   |      |  |
|-------------------|----------------|-----------|------|--|
|                   |                | RECOVERED | DIED | NOTES  |
| 5                 | 200            | 5         | 0    | Apathy and dyspnea followed by convulsive twitchings. Sloughs at injection sites |
| 5                 | 250            | 5         | 0    | As above   |
| 5                 | 300            | 5         | 0    | As above. Symptoms more marked   |
| 5                 | 350            | 2         | 3    | As above. Deaths 24 to 48 hours after administration                             |
| 5                 | 400            | 1         | 4    | As above   |

Henry,<sup>18</sup> working with Staub, attempted a clinical trial with this material, but found that man did not tolerate it. Within one and one-half hours after the oral administration of doses as small as 1.5 mg. patients complained of a sequence of disturbing side reactions. The voluntary muscles became stiff and sore, those muscles which had been fatigued by previous exercise being the first to be involved. This was followed by dyspnea and dysphagia, hyperesthesia of the skin, and lowered blood pressure. In one individual the dyspnea was

sufficiently marked to cause a feeling of suffocation of sufficient intensity as to give rise to considerable anxiety. In this patient the toxic reactions persisted for three hours. Increasing the dose caused an exacerbation of the symptoms previously described. When the level reached 20 mg., the muscle "stiffness" became acute muscular pain, the dyspnea was intensified, and nausea and vomiting occurred. The patients suffered from such intense headache that they were unable to maintain an erect posture.

Staub showed that guinea pigs may be protected from two lethal doses of parenterally administered histamine if a previous subcutaneous injection of 40 mg. per kilogram of thymoxyethyldiethylamine had been administered. We have been able to verify this observation.

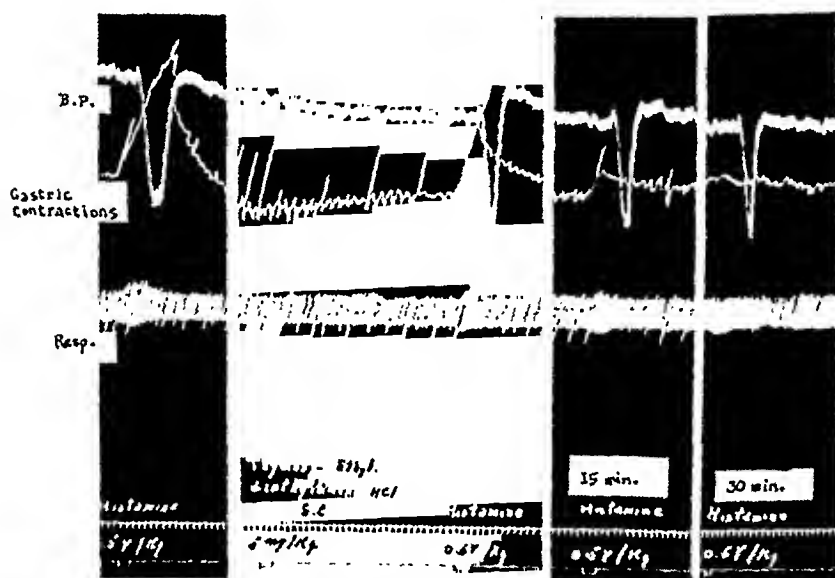


Fig. 4.—Cat under sodium barbital anesthesia. Record of carotid blood pressure, gastric contractions, and respiration. Time in hours and intervals. The fall in blood pressure and the contraction of the stomach resulting from the administration of 5 mg. of histamine per kilogram of body weight is followed by the same dose of 5 mg. per kilogram of thymoxyethylamine. The fall in the blood pressure resulting from the subsequent administration of the same dose of histamine is not affected.

The thymol derivative has no effect on histamine *in vitro*; that is, it will not combine with histamine to produce an inactive compound, nor will it convert it to an inert form. This is demonstrated by the following experiment:

Fifty milligrams of thymoxyethyldiethylamine were added to 100 c.c. of a phosphate buffer solution at pH 7.4, containing 1 mg. of histamine monohydrochloride. This system was thoroughly oxygenated and incubated at 37° C. for twenty-four hours, during which time it was under continuous agitation. At the end of this time the solution produced characteristic vasomotor responses in the intact cat, which differed in no way from those evoked by the administration of an equal quantity of untreated histamine. Under the same conditions, one unit of histaminase (Torantil) will inactivate 1 mg. of histamine monohydrochloride.

When the compound is administered subcutaneously to the intact anesthetized animal, a number of reactions occur which are dependent on the size

of the dose. Doses up to 10 mg. per kilogram produce no apparent effect. There is a slight fall in the blood pressure, but there is no effect on the respiratory rate or rhythm. The lowering of the blood pressure by small doses of histamine is not altered by this order of dose.

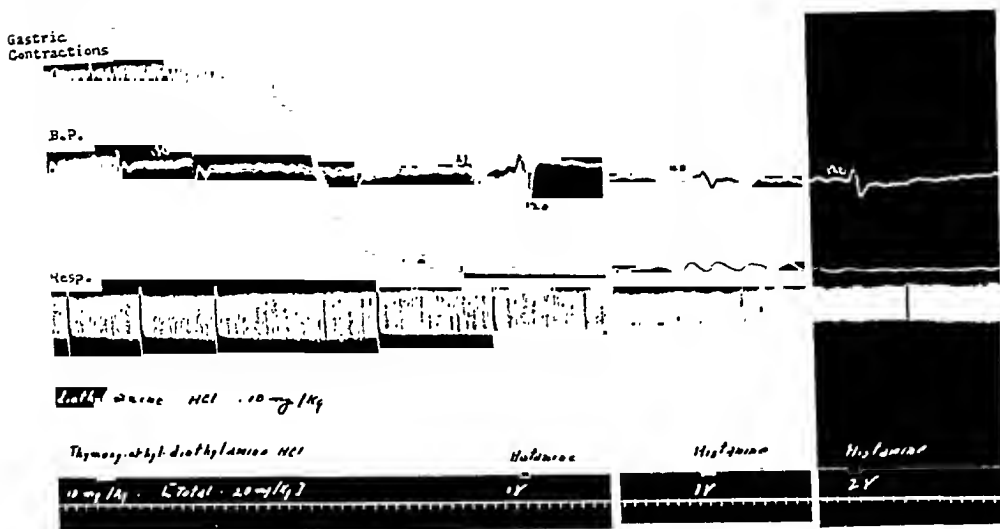


Fig. 5.—Cat under sodium barbital anesthesia. Record of gastric contractions, carotid blood pressure, and respirations. Time in five-second intervals. The administration of 20 mg. per kilogram of thymoxyethyl diethylamine causes a profound loss of tonus in the smooth muscle of the gut. The vasomotor reaction to  $1\gamma$  of histamine is markedly diminished, but not completely abolished.

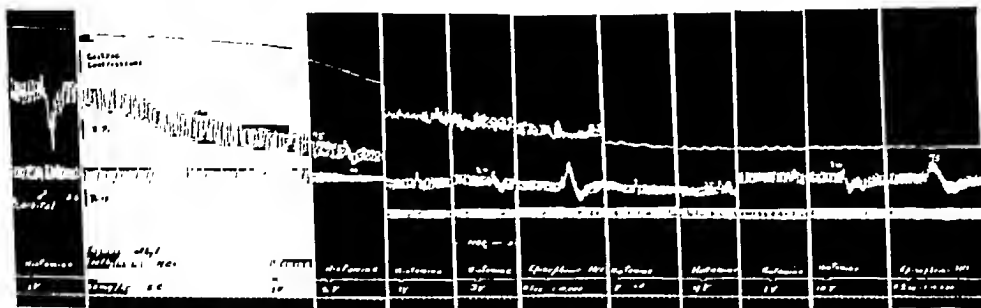


Fig. 6.—Cat under sodium barbital anesthesia. Record of gastric contractions, carotid blood pressure, and respiration. Time in five-second intervals. The normal response to  $1\gamma$  of histamine is first recorded. This is followed by the subcutaneous administration of thymoxyethyl diethylamine (30 mg. per kilogram) which causes a definite fall in the blood pressure. Within three minutes the effect of  $1\gamma$  of histamine has been abolished. One hour later the administration of 0.1 c.c. of a 1:10,000 solution of epinephrine produced no significant elevation of the blood pressure. Two hours after the administration of the thymoxyethyl diethylamine,  $10\gamma$  of histamine produced no significant fall in the blood pressure, and 0.2 c.c. of epinephrine caused no rise in the blood pressure.

Increasing the dose to 20 mg. per kilogram causes a more marked fall in the systemic blood pressure which is associated with a slight increase in the respiratory rate. The most prominent effect of doses of this order is the marked loss in tonus of the smooth muscle. At this time the reaction to standardized doses of histamine is markedly reduced, but not completely abolished.

If the dose of thymoxyethyl diethylamine is increased to 30 mg. per kilogram, the fall in the blood pressure is more marked and the effects of small doses

of histamine (1 to 5  $\gamma$ ) are almost completely obliterated. This applies not only to the fall in the systemic blood pressure normally associated with the administration of such doses of histamine, but also to its stimulating action on smooth muscle.

It should be pointed out that at this time the peripheral paralysis of the vasomotor system is so complete that it becomes impossible to evoke a normal epinephrine response.

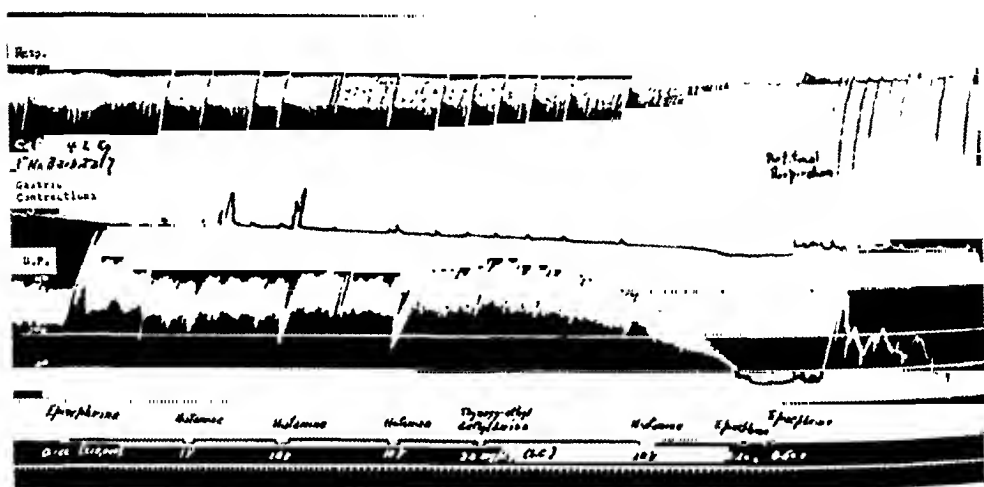


Fig. 7.—Cat under sodium barbital anesthesia. Record of respiratory movements, gastric contractions, and carotid blood pressure. (Blood pressure recorded on membrane manometer.) Time in five-second intervals. Normal responses to epinephrine and histamine are followed by the subcutaneous administration of thymoxyethyldiethylamine (30 mg. per kilogram). This causes a progressive fall in the blood pressure and results in death. There is no response to 0.1 c.c. of epinephrine (1:10,000), and a delayed, feeble response to 0.5 c.c. of epinephrine.

We have described characteristic responses to thymoxyethyldiethylamine, but these do not invariably result from the administration of this compound. We have at times seen fatal responses resulting from the subcutaneous administration of doses of this order which have not been associated with any modification of the usual histamine action on the vasomotor system.

#### SUMMARY

1. Thymoxyethyldiethylamine can inhibit the oxytocic effect of histamine *in vitro*. This action is not specific for histamine, for it will also inhibit the oxytocic effect of barium chloride.
2. Thymoxyethyldiethylamine can inhibit the fall in blood pressure in the cat resulting from the intravenous administration of histamine. Doses which are capable of producing this reaction cause a fall in the blood pressure and produce an ergotoxine-like reversal of the pressor effect of epinephrine.
3. Thymoxyethyldiethylamine can protect guinea pigs from the parenteral administration of two lethal doses of histamine.
4. Thymoxyethyldiethylamine has no inactivating effect on histamine *in vitro*.
5. Thymoxyethyldiethylamine is very toxic and highly irritant.

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## MENOPAUSE ARTHRALGIA\*

## WITH A PRELIMINARY REPORT ON THE USE OF STILBESTROL

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THE diagnosis of menopause arthralgia could well be summed up as:

"A female, fat and forty-four  
Who frowns from feverish knees,  
She 'fans in church,' her arms are sore,  
For hot flashes and flushes that freeze."

Seriously, this group of patients, whose symptoms are probably more annoying than pathologic in the early phase, do many times lead to a painful crippling condition of the weight-bearing joints later, as is borne out by histories obtained from older women who date their symptoms back to around the age of the meno-

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pause. At the present time complete clinical remissions are expected in the average patient who has received the diagnosis of menopause arthralgia. Should one of these patients fail to respond to treatment, we are more apt to question our diagnosis than the efficacy of the present established treatment.

Although the syndrome of joint and muscle pain, which occasionally accompanies or follows the artificial or the natural menopause, has never been widely accepted as a definite pathologic entity, nor the mechanism by which it takes place clearly demonstrated, the results from treatment of this syndrome certainly warrant grouping this type of rheumatic disease for special treatment. Francis Hall,<sup>1</sup> in his monograph on menopause arthralgia, has most ably described this syndrome and reports gratifying results in treatment from the use of estrogenic materials. In discussing one of Hall's papers<sup>2</sup> on this subject, I<sup>3, 4</sup> suggested the use of 10 c.c. of antohemotherapy reinforced with 101 degrees of artificial fever for one hour, together with the estrogenic materials in the treatment of those patients who fail to respond to the estrogenic materials alone. My results from the use of estrin compounds compare favorably to those reported by Dr. Hall.

#### PLAN OF TREATMENT

In the series reported here the following plan of management was applied to those carefully selected patients classed as menopause arthralgia:

##### A. General Supportive Measures:

1. Diet: Low carbohydrate, high vitamin on those patients who are overweight; otherwise a normal, well-balanced diet.
2. Avoidance of traumatic factors; this involves a myriad of things, such as general and local rest, proper shoes, avoidance of occupational strains, posture, etc.
3. Avoidance of climatic factors, such as cold, dampness, drafts, wet hands and feet, exposure, etc.
4. Foci of infections; these to be left intact throughout the active treatment or while rheumatic symptoms are present.
5. Supplementary vitamins; these to be used on patients who present a clinical deficiency. In this series the use of the vitamin B complex was most frequently indicated, especially in the group seen at the Arthritic Clinic at the Oklahoma University Hospital and the School of Medicine. Several of this group were diagnosed as having clinical pellagra.
6. Correction of disturbed or abnormal systems present, such as metabolism, gastrointestinal function, emotional stress, posture, and other pertinent factors affecting the patient's normal health.
7. Relief of pain.

##### B. "Specific" Measures:

1. In this phase the patients were divided into groups:
  - (1) Injectable estrogenic hormone used in 60 patients who received an average of 10,000 units twice weekly over an average period of from four to ten weeks.



- (2) Estrogenic hormone given by mouth or by insertion into the vagina. Thirty-four patients received 0.12 mg. in capsule form daily for an average period of four weeks.
- (3) Stilbestrol (dihydroxy alpha, beta diethylstilbene) by subcutaneous injections; 60 patients received an average dose of 0.25 mg. (7,500 international units) twice weekly for an average of from four to ten weeks.
- (4) Stilbestrol by mouth: 30 patients received 1 mg. in tablet form averaging from every two to four days.
- (5) Autohemotherapy: As mentioned, this measure was supplemented only in those patients in whom the pathology had reached an advanced degree or who had failed to respond to the estrin or stilbestrol therapy alone.
- (6) Artificial fever: As with the autohemotherapy, this measure is employed in those patients who have failed to respond to the estrin hormone or stilbestrol alone.

#### C. Physical Therapy Measures:

The same principles were applied here as in the other rheumatic phenomena. As a rule, the menopause arthritis patients have a lowered basal metabolism, and it is desirable to use measures which have a stimulating effect on the general metabolism. The more commonly used modalities are listed:

1. Heat: Local and general application. Except in the acute cases heat is usually followed with a brief application of cold. Warm baths of from fifteen to thirty minutes followed with a brief cold shower; contrast baths, hot paraffin, short wave, and hot fomentations, were helpful.

#### D. Orthopedic Measures:

1. Splinting and rest of acute areas.
2. Support where indicated. It is necessary to pay close attention to this phase of the management. It involves such features as splints, shoes, braces, and corsets. The knee is possibly one of the most important joints in that it is most frequently involved; once the disease process is started, it leads to degeneration of the joint. In our experience, the application of a leather knee cage brace reinforced with metal supports on each side hinged at the knee, has been most useful.

#### RESULTS

The results in these cases are listed in Table I. Briefly, they can be summed up as follows:

1. Sixty patients received stilbestrol by injection. In this group 80 per cent have had complete remission, 15 per cent improved, and 5 per cent failed to respond.

2. Thirty patients received stilbestrol by mouth. In this group 77 per cent had complete remission, 10 per cent improved, and 13 per cent failed to respond.

3. Thirty patients who had been receiving estrogenic hormones were changed to stilbestrol therapy. The patients were not informed of the change. Of these, 82 per cent noticed no change, 15 per cent stated they were better, and 3 per cent had increased pain.

4. Sixty patients in the control group received estrogenic hormones by injection. Of these, 85 per cent had complete remission, 10 per cent were improved, and 8 per cent failed to improve.

5. Thirty-four patients received estrogenic materials by mouth. Of these, 30 per cent had remission, 35.1 per cent were improved, and 35 per cent failed to improve.

It is interesting to note that those patients who had remission of rheumatic symptoms also had remission of the other symptoms accompanying the menopause. It is also interesting to observe that whereas the estrogenic material given by mouth failed as a whole to control the symptoms, the stilbestrol by mouth was largely adequate. Experience proved, however, that when stilbestrol is given by mouth, it is better to use 0.5 mg. instead of 1 mg., and that the tablets should be enteric coated. In the average case, the patient responded better if the stilbestrol was given daily. These measures largely eliminated the major complication, which was nausea.

TABLE I

| THERAPY USED                          | NUMBER OF PATIENTS | REMISSION            | IMPROVED             | UNIMPROVED           | COMPLICATIONS OR SIDE ACTIONS                 |
|---------------------------------------|--------------------|----------------------|----------------------|----------------------|---|
| 1. Stilbestrol by injection           | 60                 | 48 patients or 80%   | 9 patients or 15%    | 3 patients or 5%     | Nausea 17%<br>Sore breasts 5%<br>Vertigo 1.6% |
| 2. Estrin by injection                | 60                 | 49 patients or 82%   | 6 patients or 10%    | 5 patients or 8%     | Nausea 2%<br>Sore breasts 4%<br>Vertigo none  |
| 3. Stilbestrol by mouth               | 30                 | 23 patients or 77%   | 3 patients or 10%    | 6 patients or 13%    | Nausea 10%<br>Sore breasts 5%<br>Vertigo 1.6% |
| 4. Estrin by mouth                    | 34                 | 10 patients or 29.4% | 12 patients or 35.3% | 12 patients or 35.3% | None  |
| 5. Autohemotherapy with estrin        | 14                 | 12 patients or 85%   | 1 patient or 12.5%   | 1 patient or 12.5%   |   |
| 6. Combined autohemotherapy and fever | 15                 | 12 patients or 80%   | 1 patient or 6%      | 2 patients or 14%    |   |

## DISCUSSION

The stilbene derivatives have been under careful experimental observation for the past six years. In 1934 Cook, Dodds, Hewett, and Lawson<sup>5</sup> described the estrogenic activity of a series of phenanthrene and dibenzanthracene compounds. It was shown that these substances can replace estrone in every way. In 1938 Dodds and Lawson<sup>6</sup> demonstrated that dihydroxystilbene had definite estrogenic activity.

More investigations were continued and the potent substance, 4,4; dihydroxydiethylstilbene (diethylstilbene stilbestrol), was discovered.<sup>7-10</sup>

Extensive pharmacologic tests of stilbestrol have been carried out on experimental animals, and the following facts have been established:

1. Geschickter,<sup>11</sup> in 1939, in discussing mammary carcinoma in the rat with metastasis induced by estrogen, suggests that "the mammary cancers induced in the rats by the injection of estrogenic substances are due to the physiologic changes produced rather than to the cancerogenic nature of the chemicals used."

2. Selye,<sup>12</sup> in 1939, in reporting on the toxicity of stilbestrol, concluded: "Experiments on the mouse and the rat indicate that when given in very high doses both estradiol and diethyl stilbestrol may exert toxic effects. In the mouse, death may occur under the influence of such estrogens."

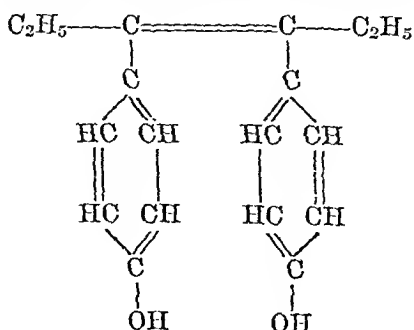
3. Loeser,<sup>13</sup> in 1939, in reporting on the clinical effect of diethylstilbestrol, states that this product has a marked substitution action in various forms of ovarian insufficiencies, but that 6 of the 15 patients in whom it was used had nausea or vomiting.

4. Others, MacGregor,<sup>14</sup> Bishop, Boycott, and Zuckerman,<sup>15</sup> Winterton,<sup>16</sup> Kellar and Sutherland,<sup>17</sup> Rees,<sup>18</sup> Karnaky,<sup>19</sup> Ehrhardt, Kramann, and Schäfer<sup>20</sup> in 1939 reported favorably on the use of stilbestrol. Karnaky<sup>19</sup> states that it is as potent when given by mouth as by injection and that no toxic effects were felt. Varanget,<sup>21</sup> reporting in the 1939 literature, however, advises against its use.

In the 1940 literature 33 papers were published from January to June. Of these, I was able to obtain 15. In these, 12 of the authors<sup>22-33</sup> advise the use of stilbestrol, while 3 of them<sup>34-36</sup> advise against its use. The universal objection is chiefly the side action of nausea and epigastric pain.

In our series 10 patients (17 per cent) complained of nausea. In all these, however, the drug was continued at a reduced dosage. In only one patient was it necessary to discontinue the drug. In using this preparation, however, all the factors contraindicating the use of the estrins must be closely observed and followed.

The terminology of stilbestrol is somewhat confusing due to the synonyms used. Diethylstilbestrol, 5,5, dihydroxydiethylstilbene, and diethylstilbene are used. The empirical formula is  $C_{18}H_{20}O_2$ . The structural formula:



It is a white, odorless, tasteless, nonhygroscopic powder. Less than 1 mg. will go into solution in 20 c.c. of water. It is soluble in most organic solvents and in vegetable oils. It is soluble in alkali hydroxides.

## CONCLUSIONS

1. Forty-eight patients (80 per cent) diagnosed as having menopause arthralgia had satisfactory remissions following the injections of stilbestrol used in conjunction with other supportive measures.

2. Forty-nine patients (82 per cent) as controls responded satisfactorily to estrin injections along with the same supportive measures.

3. In 30 patients stilbestrol was successfully substituted for estrin with only 3 per cent having return of original symptoms.

4. Twenty-three patients (77 per cent) receiving stilbestrol by mouth had clinical remission as compared to 10 patients (30 per cent) who received estrin that responded by clinical remission.

If the foregoing findings are borne out by later investigators, and if complications not yet detected in the patient do not make their appearance, stilbestrol should supplant the use of the estrogenic hormone for two reasons: (1) It is effective when given orally. (2) The cost to the patient is greatly reduced. In considering the fact that the treatment is largely substitutional and must be continued for a comparatively long period of time, these two factors would justify the use of stilbestrol.

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## CARDIAC DEPRESSION BY MERCURIAL DIURETICS\*

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**I**N VIEW OF the fact that mercurial diuretics are often used in cases of edema and dropsy of cardiac origin, these experiments were performed on isolated turtle hearts to ascertain whether impaired cardiac action may follow their use in concentrations which prevail therapeutically, and to note the antidotal effect of sodium thiosulfate and certain cardiac stimulants.

A review of the abundant literature is not intended, but certain papers are cited for their pertinent interest. Salant and Kleitman<sup>1</sup> in 1922 studied the action of mercurial salts on the hearts of intact cats and dogs, and on the isolated hearts of frogs and turtles. Turtle hearts developed delirium cordis after twelve minutes of perfusion with mercuric chloride, one part in one million. This persisted for more than twenty minutes during subsequent perfusion with Ringer's solution. Similar effects were observed on the hearts of cats under urethane anesthesia, but of shorter duration. Wolf<sup>2</sup> in 1932, Molnar<sup>3</sup> in 1935, and Greenwald and Jacobson<sup>4</sup> in 1937, report cases of sudden death following the administration of mercurial diuretics to patients. Deaths following the

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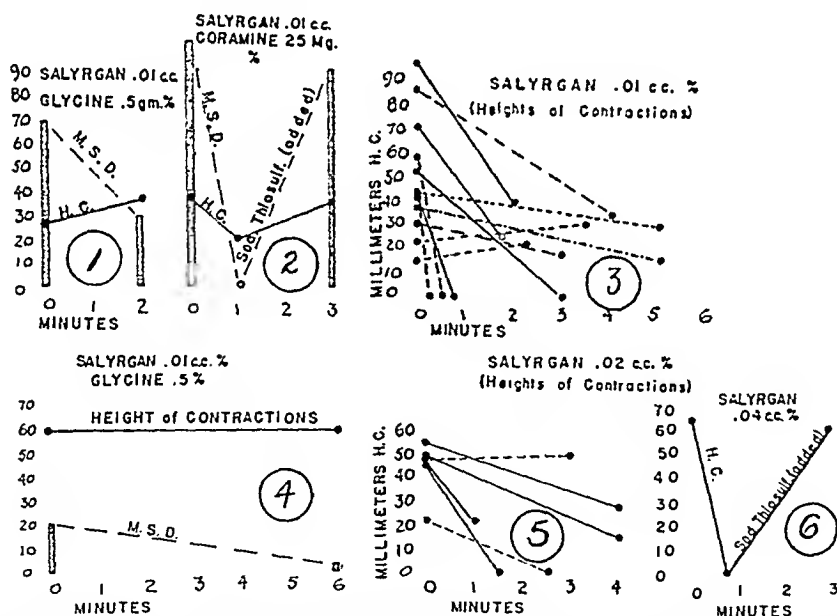
therapeutic administration of calomel are reported by Backer<sup>5</sup> in 1921, Burnet and Pestal<sup>6</sup> in 1924, and Póvoa<sup>7</sup> in 1927.

### METHOD

Ringer's solution with the pH adjusted between 7.2 and 7.4 served as the vehicle of perfusion for all drugs used. The apparatus and technique were the same as used in previous publications.<sup>8</sup> Solutions of the mercurial diuretics, salyrgan and mereupurin, were prepared by diluting the 1 c.c. commercial ampoules. The most frequently used concentration in this study, 0.01 c.c. per 100 c.c. of Ringer's solution, had a mercury content of one part in 300,000.

### RESULTS

In the charts the ordinates indicate by black dots the heights of cardiac contractions as written by the lever on the smoked drum, while the vertical columns indicate relatively the cardiac work done in terms of stroke distance per minute (M.S.D.); the figures in the abscissa indicate the perfusion time in minutes.



Figs. 1-6.—Fig. 1 shows height of contractions increased by glycine in the presence of salyrgan, 0.01 c.c. per 100 c.c. of Ringer's solution, but due to the slowed heart rate caused by salyrgan the cardiac work (M.S.D.) is decreased.

Fig. 2, both the height of contractions and the rate are decreased by salyrgan, 0.01 c.c. per 100 c.c., in the presence of coramine, but sodium thiosulfate causes immediate recovery.

Fig. 3, salyrgan, 0.01 c.c. per 100 c.c., decreased the height of contractions in 9 of 11 experiments.

Fig. 4, another experiment similar to Fig. 1.

Fig. 5, height of contractions decreased in 5 of 6 experiments by salyrgan, 0.02 c.c. per 100 c.c.

Fig. 6, salyrgan, 0.04 c.c. per 100 c.c., quickly brought height of contractions to zero; added sodium thiosulfate caused immediate recovery.

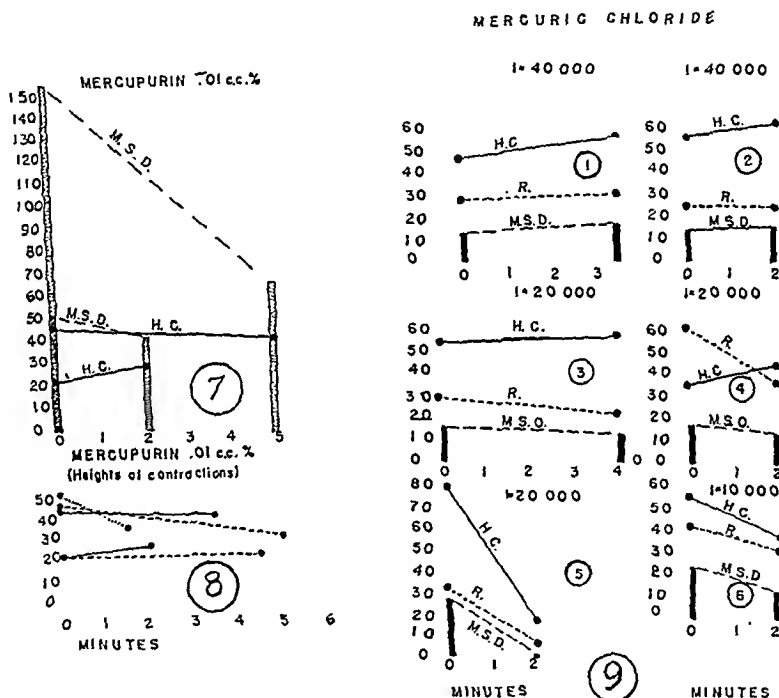
**Salyrgan.** Figs. 1 and 4 show that salyrgan perfused in a concentration of 0.01 c.c. per 100 c.c. in the presence of a cardiac stimulant (glycine 0.5 Gm. per cent) increased the height of contractions but decreased the cardiac work (M.S.D.).

Fig. 2 indicates that both the height of contractions and the M.S.D. are decreased by salyrgan, 0.01 c.c. per 100 c.c., in the presence of another cardiac stimulant (coramine 25 mg. per cent). Recovery is prompt with the addition of sodium thiosulfate, 10 mg. per cent, to the perfusate.

Fig. 3 shows 11 experiments wherein the same concentration of salyrgan was perfused. In nine of these, or 82 per cent, the height of contractions was decreased.

Fig. 5 shows five experiments wherein 0.02 c.c. per cent of salyrgan was perfused. In four of these, or 80 per cent, the height of contractions was decreased.

Fig. 6 shows the decrease in height of contractions caused by perfusion with salyrgan, 0.04 c.c. per cent, and prompt recovery when sodium thiosulfate was added.



Figs. 7-9.—Fig. 7 shows effect of mercurpurin, 0.01 c.c. per 100 c.c. of Ringer's solution, on height of contractions and cardiac work (M.S.D.).

Fig. 8 shows heights of contractions in 5 experiments wherein mercurpurin, 0.01 c.c. per 100 c.c. was perfused.

Fig. 9 shows graphs of 6 perfusion experiments with bichloride of mercury in concentrations of 1:40,000 to 1:10,000.

**Mercupurin.** Fig. 7 shows the results of mercurpurin, 0.01 c.c. per cent, on the height of contractions and the minute stroke distance. In both experiments the amount of work done (M.S.D.) was decreased, but not to the same extent. In one the height of contractions was increased.

Fig. 8 shows the heights of contractions of five experiments on hearts perfused with the same concentration of mercurpurin.

**Mercuric Chloride.** Fig. 9, graphs 1 to 6 inclusive, shows for comparison the effect of perfusion of the isolated turtle heart with bichloride of mercury in the concentrations of 1:40,000, 1:20,000, and 1:10,000 in Ringer's solution.

The concentration of 1:40,000 slightly increased the height of contractions and the rate with a consequent increase in the M.S.D. The susceptibility of the individual hearts varied, e.g., the concentration of 1:20,000, graph 5, in one heart was more depressing than that of 1:10,000, chart 6, in another heart.

**Mercurous Chloride.** Fig 10A is the second tracing of a heart tracing showing fully developed delirium cordis by perfusion with calomel in a concentration of 1:10,000, shaken and decanted, and the decanted fluid used as perfusate. When Ringer's solution was subsequently perfused, the irregularity persisted for several minutes, the mercury being difficult to wash out; but when sodium thiosulfate was perfused, the antidotal effect was immediate.

Fig. 10B is a specimen tracing from which the charts of Fig. 2 were derived. Notwithstanding the presence of coramine, 25 mg. per 100 c.c. with the mercurial diuretic, there was typical mercury depression evidenced by decrease in height of contractions followed by partial, then complete, heart block. Continuation of perfusion with the same solution plus sodium thiosulfate, 10 mg. per 100 c.c., resulted in immediate recovery. These tracings, 10A and 10B, are shown because they typically illustrate the cohesion of mercury salts in the cardiac tissues, and that sodium thiosulfate rapidly penetrates the tissues performing its well-known antidotal action.

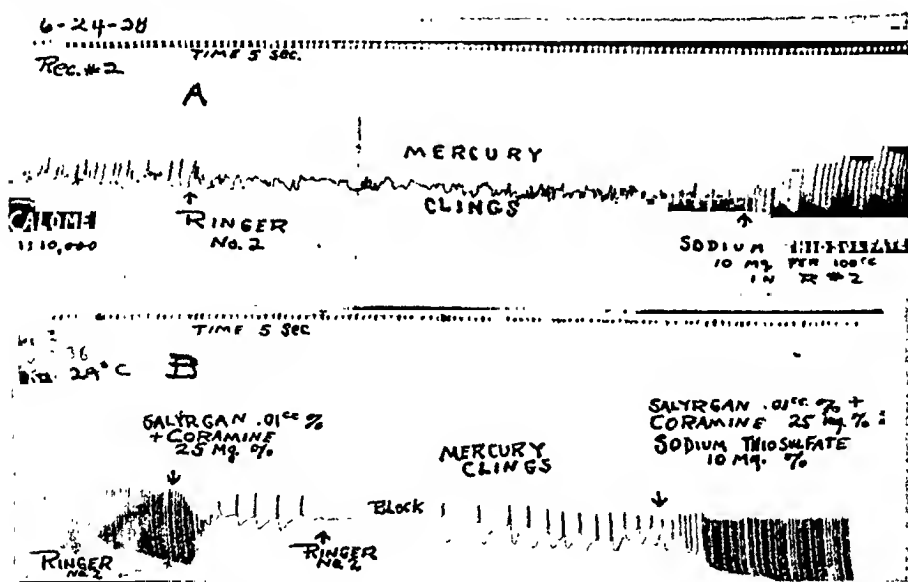


Fig. 10.—A (upper chart) shows the heart tracing after the full development of delirium cordis by perfusion with decanted Ringer's solution in which calomel 1:10,000 had been shaken. The mercury effect was not readily removed by perfusion with Ringer's solution until sodium thiosulfate was added; whereupon recovery was immediate. B (lower chart) is the experiment from which the graph in Fig. 2 was mathematically derived.

#### COMMENT

That the mercurial diuretics occupy a position of great usefulness in the treatment of selected cases of edema and dropsy cannot be denied. It is desired to emphasize that their effectiveness is due to their mercury content, and that their toxicity is not appreciably different from that of a similar concentration of mercury in other forms. No claim is made here that tolerance to mercury is



identical in the different species insofar as the cardiac tissues are concerned, nor is this research intended to discourage the proper use of the mercurial diuretics, under close medical supervision, in suitable cases.

### CONCLUSIONS

A study was made of the toxic effects of mercurial diuretics and the protective effects of various heart stimulants on isolated turtle hearts.

1. The mercurial diuretics possess a toxicity to the heart with reference to their mercury content that is not essentially different from that of inorganic mercury compounds. This side action is apart from diuresis and may sometimes impede it.

2. The purine derivatives especially, and to some extent certain of the other cardiac stimulants, when used in conjunction with mercurial diuretics, somewhat decreased the toxicity to the heart when the dose of the latter was sufficiently small to be of low toxicity. These cardiac stimulants, however, had relatively little antidotal action.

3. Sodium thiosulfate perfused through the mercury-poisoned heart in complete block rapidly penetrates the tissues and restores the normal force and rhythm whether the toxic agent is one of the mercurial diuretics, or calomel, or bichloride of mercury. This antidotal action takes place even if the mercury compound is continuously perfused in the presence of the antidote.

Thanks are extended to Dr. Martin H. Fischer for constructive criticism and suggestions during the conduct of this research.

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## THE EFFECT OF SUPPLEMENTAL AMYLASE ON DIGESTION\*

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JUDGING from the comments in many textbooks of materia medica and therapeutics, it must be concluded that, at the present time, most authors question the value of enzyme therapy. This is probably due, at least in part, to a report by Bastedo.<sup>1</sup> He submitted a questionnaire pertaining to the value of digestive enzymes, to a representative group of gastroenterologists. On the basis of their replies, he was forced to conclude that at the time of his survey, these agents were of minor importance in therapeutics.

Two widely held conceptions appear to be largely responsible for the low esteem in which enzyme therapy is held. It is generally believed that (a) deficient digestion is rarely, if ever, a cause of symptoms, and that (b) in most cases enzyme therapy is necessarily doomed to failure because the administered enzyme would be quickly inactivated by the acid gastric juice. That both of these concepts are erroneous has been established conclusively. It has been shown repeatedly in laboratory animals in which pancreatic juice has been prevented from reaching the intestine that enzyme therapy exerts a potent effect on the quantity of food assimilated.<sup>2</sup> Silverman, Denis, and Leeche<sup>3</sup> have shown that a number of different pancreas preparations augment the concentration of enzymes in the intestine, and Ivy, Schmidt, and Beazell<sup>1</sup> have shown that, when properly administered, approximately 85 per cent of ingested enzyme reaches the intestine in an active form. More recently, Beazell, Schmidt, and Ivy<sup>2</sup> have discussed the value of enzyme therapy in the treatment of achylia pancreatica. In 4 patients suffering from a complete suppression of the external secretion of the pancreas, they found that adequate enzyme therapy reduced the quantity of fat and nitrogen eliminated in the feces by an average of 63.3 per cent and 62 per cent, respectively. Further, the clinical response of the patients was gratifying in all cases.

The investigation herein discussed was undertaken to test further the potentialities of enzyme therapy. It was instituted to provide answers to two problems: (1) What is the effect of supplementary diastase on starch digestion in the stomach? (2) Is that fraction of the supplementary diastase which reaches the intestine in an active form capable of effective hydrolysis? The first of these two problems has already been attacked by Ivy, Schmidt, and Beazell.<sup>4</sup> They were primarily concerned with the effect of diastase on the rate of digestion, rather than on the total quantity of starch digested. Furthermore, in most of their studies they employed a cereal (farina) as a test meal, so their observations cannot be unequivocally used as a basis for anticipating

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the effect of supplementary diastase in the presence of a mixed meal. For these reasons, it appeared desirable to repeat and extend their experiments.

For a number of reasons taka-diastase\* was chosen as the test enzyme preparation. First, it is a potent diastatic ferment having an activity of 0.96 Willstätter<sup>5</sup> and 11,260 Davison<sup>6</sup> units per gram; second, it is active over a relatively wide pH range; and third, in powder form it can be stored indefinitely without alteration in activity.

#### EFFECT OF TAKA-DIASTASE ON GASTRIC DIGESTION IN NORMAL HUMAN BEINGS

*Methods.*—The details of the experimental methods and calculations have been described and discussed in a previous communication.<sup>7</sup> Briefly, the experiment consisted of feeding normal young adult males a meal of known composition, withdrawing it from the stomach at the end of a specified period of time, and determining the ratio of "digested" to "undigested" carbohydrate and, in certain experiments, protein in the recovered material. The effect of the taka-diastase was determined by comparing the results of one or more control experiments with one or more in which taka-diastase was taken with the standard meal.

Twelve clinically normal young adult males were used as subjects for the experiment. In the initial phases a test meal consisting of 150 Gm. of beef steak, 150 Gm. of boiled potatoes, 50 Gm. of green peas, 10 Gm. of butter, and 200 c.c. of water was employed. When used, 2 Gm. of taka-diastase were dissolved in the water which was sipped throughout the meal. Five persons who were capable of voluntarily regurgitating the gastric contents for analysis were tested with this meal. During preliminary experiments it became apparent that under the conditions of the experiment taka-diastase effected no augmentation of gastric starch digestion. Apparently in these normal healthy human beings, the ingestion of an appetizing meal stimulated the flow of sufficient saliva to effect maximum digestion so that supplementary amylase had no demonstrable effect. Therefore, in order to determine whether taka-diastase was capable of hydrolyzing starch in the stomach, it was necessary to have the subjects voluntarily induce a salivary deficiency. This was accomplished by having them "bolt" the meal, and then during the hour the meal remained in the stomach, swallow as little saliva as possible.

Subsequently, this meal was abandoned in favor of a finely divided homogeneous meal for the following reasons: (a) Only a limited number of subjects were capable of cooperating in an experiment requiring regurgitation of the meal. (b) By resorting to a finely divided meal it was possible to evacuate completely the stomach by means of an Ewald tube, and to (c) determine the dilution of the meal by the gastric juice. This latter determination made it possible to calculate the fraction of the ingested taka-diastase remaining in the stomach in an active form at the termination of the experiment, and the fraction of the digested material recovered from the stomach that represented pre-formed digested material contributed by the meal.

\*Product of Parke, Davis & Co.

The finely divided meal consisted of 100 Gm. of ground lean beef and 30 Gm. of potato starch boiled in sufficient water to yield a final volume of 400 c.c. Twenty milligrams of phenolsulfonephthalein was dissolved in the water used in preparing the meal. The dilution of the dye served to determine the dilution of the meal by the gastric juice and saliva. When this meal was employed, it was unnecessary to induce a state of artificial salivary deficiency in order to demonstrate augmentation with taka-diastrase.

*Results.*—The results obtained with the unground meal are summarized in Table I. Of the 5 persons tested, 3 showed a decided increase in the quantity of starch digested in the stomach when taka-diastrase was added to the meal, and the remaining 2 showed no change. The results are significant, since whenever a change occurred it was always in favor of more complete digestion when taka-diastrase was taken with the meal.

TABLE I

EFFECT OF TAKA-DIASTASE ON GASTRIC DIGESTION OF THE STARCH IN A NORMAL MEAL  
(Two grams of taka-diastrase were used. Normal subjects voluntarily induced salivary deficiency.)

| SUB-<br>JECT | COMPOSITION<br>OF MEAL     |      |                 |      | MATERIAL RECOVERED<br>FROM STOMACH |     |     |     |                            |       | TOTAL<br>SUGAR* |      | % DIGES-<br>TION |      |
|--------------|----------------------------|------|-----------------|------|------------------------------------|-----|-----|-----|----------------------------|-------|-----------------|------|------------------|------|
|              | TOTAL<br>CARBO-<br>HYDRATE |      | TOTAL<br>SUGAR* |      | VOLUME                             |     | pH  |     | TOTAL<br>CARBO-<br>HYDRATE |       |                 |      |                  |      |
|              | C                          | T    | C               | T    | C                                  | T   | C   | T   | C                          | T     | C               | T    | C                | T    |
| J. W.        | 44.8                       | 52.4 | 8.4             | 13.9 | 85                                 | 110 | 3.9 | 3.9 | 4.08                       | 2.86  | 1.73            | 1.76 | 23.7             | 36.4 |
| L. R.        | 47.6                       | 49.6 | 10.1            | 12.1 | 100                                | 125 | 4.2 | 4.8 | 3.40                       | 4.00  | 2.02            | 2.50 | 38.3             | 38.2 |
| T. K.        | 36.4                       | 42.6 | 6.8             | 7.9  | 155                                | 125 | 4.0 | 3.6 | 5.78                       | 5.05  | 2.32            | 2.75 | 21.5             | 35.1 |
| H. A.        | 44.8                       | 52.4 | 8.4             | 13.9 | 385                                | 270 | 3.7 | 4.0 | 21.60                      | 14.60 | 8.17            | 6.80 | 18.9             | 20.0 |
| J. C.        | 36.4                       | 42.6 | 6.8             | 7.9  | 225                                | 325 | 4.1 | 3.5 | 8.10                       | 9.56  | 4.41            | 7.15 | 35.7             | 55.5 |
| Aver-<br>age |                            |      |                 |      |                                    |     |     |     |                            |       |                 |      | 27.6             | 36.7 |

\*As maltose.

The results obtained with the finely divided meal are summarized in Tables II and III. Taka-diastrase had no effect on the digestion of protein, which is not surprising since it possesses only feeble proteolytic activity.

In the case of starch digestion, on the other hand, taka-diastrase effected a significant augmentation in all persons with a 2 Gm. dose, and in at least 5 of the 8 persons with a 1 Gm. dose. In the cases in which duplicate control determinations were conducted, satisfactory checks were obtained in all persons except one. This one was also erratic in his response to taka-diastrase, a greater effect being obtained with 1 Gm. than with 2 Gm. It should be noted, however, that in the presence of taka-diastrase digestion was more complete than in either of the control experiments.

In no case did a significant fraction of the taka-diastrase remaining in the stomach retain its activity.

#### EFFECT OF TAKA-DIASTASE ON GASTRIC DIGESTION IN THE DOG

Having established that conditions in the human stomach are not incompatible with effective hydrolysis by taka-diastrase, it became desirable to study its action under circumstances which made it possible to determine total gastric

TABLE II  
EFFECT OF TAKA-DIASTASE ON THE GASTRIC DIGESTION OF STARCH IN THE NORMAL HUMAN SUBJECT  
(Finely divided test meal)

| SUB-<br>JECT | VOLUME<br>RECOVERED<br>FROM<br>STOMACH |                |                | RECOVERED<br>VOLUME<br>CORRECTED<br>FOR<br>DILUTION |                |                | pH<br>RECOVERED<br>MATERIAL |                |                | SUGAR*<br>RECOVERED<br>FROM<br>STOMACH<br>(AS MALTOSE) |                |                | STARCH†<br>EQUIVALENT<br>OF RECOVERED<br>SUGAR |                |                | CALCULATED<br>STARCH<br>CONTENT OF<br>RECOVERED<br>MATERIAL |                |                | PER CENT<br>OF STARCH<br>DIGESTED<br>TO MALTOSE |                |                |
|--------------|--|----------------|----------------|---|----------------|----------------|-----------------------------|----------------|----------------|--|----------------|----------------|--|----------------|----------------|---|----------------|----------------|---|----------------|----------------|
|              | C                                      | T <sub>1</sub> | T <sub>2</sub> | C   | T <sub>1</sub> | T <sub>2</sub> | C                           | T <sub>1</sub> | T <sub>2</sub> | C  | T <sub>1</sub> | T <sub>2</sub> | C  | T <sub>1</sub> | T <sub>2</sub> | C   | T <sub>1</sub> | T <sub>2</sub> | C   | T <sub>1</sub> | T <sub>2</sub> |
| H. W.*       | 380                                    | 455            | 450            | 324   | 338            | 304            | 2.2                         | 3.7            | 2.2            | 4.9  | 6.9            | 4.7            | 4.5  | 5.5            | 4.4            | 24.3  | 22.8           | 25.4           | 18.5  | 24.1           | 17.4           |
| J. W.*       | 250                                    | 310            | 350            | 227   | 282            | 282            | 1.7                         | 2.7            | 2.8            | 3.0  | 7.8            | 4.1            | 2.8  | 6.2            | 3.9            | 17.0  | 21.2           | 21.9           | 16.5  | 20.2           | 17.6           |
| R. M.*       | 285                                    | 290            | 205            | 170   | 143            | 236            | 2.6                         | 2.5            | 3.3            | 2.3  | 4.9            | 2.7            | 2.1  | 3.9            | 2.5            | 12.8  | 17.7           | 10.7           | 16.4  | 22.0           | 23.6           |
| O. J.        | 235                                    | 285            |                | 188   |                | 235            | 1.9                         | 2.8            |                | 3.7  | 6.4            |                | 3.4  | 5.9            |                | 14.1  | 17.6           |                | 24.1  | 33.5           |                |
| W. L.*       | 330                                    | 400            | 340            | 207   | 263            | 333            | 2.5                         | 2.9            | 2.6            | 4.0  | 7.6            | 6.1            | 3.7  | 7.0            | 5.7            | 15.5  | 25.0           | 19.7           | 24.0  | 28.0           | 30.8           |
| M. R.*       | 100                                    | 100            | 135            | 99  | 123            | 133            | 2.4                         | 2.6            | 2.8            | 2.1  | 3.6            | 3.0            | 1.9  | 3.4            | 2.8            | 7.4   | 9.9            | 9.2            | 25.7  | 34.4           | 33.0           |
| J. W.*       | 185                                    | 145            | 125            | 163   | 99             | 118            | 2.6                         | 3.3            | 3.3            | 3.4  | 3.8            | 2.0            | 3.1  | 3.6            | 1.9            | 12.2  | 8.9            | 7.4            | 25.4  | 40.5           | 27.5           |
| G. L.*       | 257                                    | 350            | 310            | 299   | 239            | 246            | 1.5                         | 1.8            | 5.4            | 4.3  | 8.1            | 9.5            | 4.0  | 7.5            | 8.9            | 17.1  | 17.9           | 18.4           | 22.4  | 41.8           | 45.2           |
| V. S.        | 215                                    |                | 260            | 179   | 142            |                | 1.7                         |                | 1.8            | 2.4  |                | 4.0            | 2.2  |                | 3.7            | 13.4  | 10.7           |                | 16.4  | 34.7           |                |
| Aver-<br>age | 250                                    | 296            | 272            | 198   | 235            | 206            | 2.1                         | 2.8            | 3.0            | 3.3  | 6.1            | 4.5            | 3.0  | 5.40           | 4.23           | 14.8  | 17.6           | 15.4           | 20.2  | 30.7           | 27.5           |

\*Corrected for preformed sugar from meal and taka-diastase.

†Sugar  $\times 0.925$ .

\*Control values represent average of two determinations.

TABLE III  
EFFECT OF TAKA-DIASE ON GASTRIC DIGESTION OF PROTEIN BY NORMAL HUMAN SUBJECTS  
(Finely divided meat)

| SUBJECT | VOLUME RECOVERED FROM STOMACH |     | RECOVERED VOLUME CORRECTED FOR DILUTION |     | pH RECOVERED MATERIAL |      | TOTAL NITROGEN INGESTED |      | TOTAL SUBPROTEOSE NITROGEN INGESTED |       | TOTAL NITROGEN RECOVERED |       | TOTAL SUBPROTEOSE NITROGEN RECOVERED |       | PER CENT OF INGESTED NITROGEN EVACUATED FROM STOMACH |      | % DIGESTION OF NITROGEN REMAINING IN STOMACH* |      |
|---------|-------------------------------|-----|---|-----|-----------------------|------|-------------------------|------|-------------------------------------|-------|--------------------------|-------|--------------------------------------|-------|--|------|---|------|
|         | C                             | T   | C                                       | T   | C                     | T    | C                       | T    | C                                   | T     | C                        | T     | C                                    | T     | C  | T    | C   | T    |
| J. W.   | 230                           | 125 | 197                                     | 99  | 2.6                   | 3.3  | 3.04                    | 3.41 | 0.336                               | 0.304 | 1.23                     | 0.470 | 0.202                                | 0.085 | 59.5   | 86.0 | 3.0   | 2.10 |
| H. W.   | 440                           | 450 | 366                                     | 338 | 1.8                   | 2.2  | 2.45                    | 3.41 | 0.336                               | 0.304 | 1.63                     | 1.69  | 0.317                                | 0.292 | 33.4   | 50.5 | 0.55  | 2.10 |
| R. M.   | 230                           | 205 | 144                                     | 143 | 3.5                   | 3.3  | 2.98                    | 2.63 | 0.312                               | 0.304 | 0.930                    | 0.758 | 0.076                                | 0.096 | 69.0   | 72.0 | 0   | 0    |
| J. Wa.  | 215                           | 350 | 140                                     | 292 | 1.8                   | 2.8  | 2.98                    | 2.45 | 0.312                               | 0.336 | 0.615                    | 1.79  | 0.093                                | 0.315 | 79.4   | 27.0 | 0   | 3.90 |
| G. D.   | 270                           | 310 | 211                                     | 246 | 1.4                   | 5.4  | 2.63                    | 2.43 | 0.304                               | 0.336 | 0.698                    | 0.857 | 0.173                                | 0.285 | 73.5   | 71.8 | 1.85  | 9.45 |
| V. S.   | 215                           | 260 | 179                                     | 142 | 1.7                   | 1.8  | 2.63                    | 2.43 | 0.304                               | 0.336 | 0.636                    | 0.518 | 0.138                                | 0.148 | 75.8   | 78.8 | 0.31  | 5.80 |
| W. L.   | 340                           | 340 | 188                                     | 263 | 2.5                   | 2.6  | 2.39                    | 2.77 | 0.304                               | 0.344 | 0.71                     | 1.410 | 0.190                                | 0.301 | 70.0   | 49.9 | 6.6   | 5.7  |
| M. B.   | 58                            | 125 | 38                                      | 123 | 2.1                   | 2.8  | 2.39                    | 2.77 | 0.304                               | 0.344 | 0.194                    | 0.443 | 0.042                                | 0.124 | 92.0   | 86.0 | 6.7   | 4.1  |
| Average | 250                           | 272 | 183                                     | 206 | 2.18                  | 3.02 | 2.60                    | 2.78 | 0.314                               | 0.326 | 0.831                    | 0.988 | 0.151                                | 0.206 | 69.0   | 65.0 | 2.4   | 4.2  |

\*Corrected for preformed soluble nitrogen contributed by meat.

digestion. In the studies on the human subjects only the state of digestion at the end of a specified period of time could be determined. In order to determine total digestion in the stomach, a duodenostomy or duodenal fistula must be present so that the total gastric discharge can be collected for analysis as it is evacuated through the pylorus. Therefore, in 3 dogs the bile was diverted to the lower jejunum by means of a cholecystojejunostomy, the pancreatic juice was prevented from reaching the intestine by resecting the portion adherent to the duodenum, and a duodenostomy was created. By introducing a large catheter (28 F) into the duodenum through the fistula, it was possible to collect the uncontaminated gastric contents as they were evacuated through the pylorus. Since the dog's saliva contains no ptyalin, results in the dog might, with justification, be used to predict what would occur in the ptyalin-deficient patient.

*Methods.*—The meal used in these experiments was identical to the finely divided meal used in the experiments on human beings. In the case of one animal it was necessary to dilute the meal and administer it by means of a stomach tube. In the experiments in which taka-diastase was tested, 2 Gm. were mixed with the meal immediately before administration. Continuous collection of the discharge from the fistula was instituted immediately after the meal was ingested and continued until the stomach was empty. The material was permitted to accumulate in the collecting receptacle for periods of thirty minutes before being sampled for analysis. The analytical methods have been described and discussed previously.<sup>7</sup>

*Results.*—The results of this experiment are summarized in Table IV. In the case of each animal the results represent an average of three control determinations and three determinations with taka-diastase. It is unnecessary to record the results of individual experiments, since the variations were inconsequential. These results reveal the following facts: (1) The fraction of the ingested starch digested in the stomach was markedly augmented by taka-diastase in all cases. The average for the 3 animals was 7.58 per cent digestion without taka-diastase, and 38.9 per cent digestion with taka-diastase. These values demonstrate that in the absence of ptyalin, starch digestion in the stomach is of no significance, and that adequate substitution therapy will reduce a large fraction of the starch to simple sugars. (2) The fraction of ingested taka-diastase reaching the intestine in an active form is subject to wide individual variation, being dependent on the rate of secretion of acid by the stomach. In animal No. 2, in which the fall in pH approaches that in man, the diastase was almost entirely inactivated in the stomach. It must be concluded that when administered in this manner the effect of taka-diastase is confined almost entirely to the period during which it remains in the stomach. (3) The addition of taka-diastase to the test meal had no significant effect on the rate of emptying of the stomach.

#### EFFECT OF TAKA-DIASTASE ON STARCH DIGESTION IN THE SMALL INTESTINE

Having demonstrated that taka-diastase has a positive and significant effect on starch digestion in the stomach, the effect of that fraction that escapes inactivation by the gastric juice was investigated.

TABLE IV

| DOG                   | VOLUME OF FLUID RECOVERED | RECOVERED VOLUME CORRECTED FOR DILUTION | TIME (HR.)   | ACID                           |                                  | SUGAR RECOVERED (AS MALTOSE) | STARCH EQUIVALENT OF RECOVERED SUGAR | CALCULATED STARCH OF RECOVERED FLUID | PER CENT OF INGESTED STARCH DIGESTED IN STOMACH | DIASTASE RECOVERED (MG. OF TAKA*) | % OF INGESTED TAKA EVACUATED IN ACTIVE FORM |
|-----------------------|---------------------------|---|--|--------------------------------|----------------------------------|------------------------------|--------------------------------------|--------------------------------------|---|-----------------------------------|---|
|                       |                           |   |  | FREE                           | TOTAL                            |                              |                                      |                                      |   |                                   |   |
| 1                     |                           |   |  |                                |                                  |                              |                                      |                                      |   |                                   |   |
| Control               | 169                       | 115                                     | $\frac{1}{2}$<br>1<br>1 $\frac{1}{2}$<br>2                         | 0<br>0<br>0<br>0               | 44<br>44<br>52<br>52             | 0.45                         | 0.42                                 | 8.6                                  | 4.85  | 0                                 |   |
| Taka-diastase (2 Gm.) | 198                       | 165                                     | $\frac{1}{2}$<br>1<br>1 $\frac{1}{2}$<br>2                         | 0<br>0<br>0<br>0               | 26<br>37<br>47<br>57             | 6.59                         | 6.15                                 | 12.4                                 | 49.5  | 513                               | 25.6  |
| 2                     |                           |   |  |                                |                                  |                              |                                      |                                      |   |                                   |   |
| Control               | 442                       | 356                                     | $\frac{1}{2}$<br>1<br>1 $\frac{1}{2}$<br>2<br>2 $\frac{1}{2}$<br>3 | 0<br>0<br>10<br>20<br>25<br>25 | 40<br>59<br>64<br>72<br>75<br>80 | 1.7                          | 1.6                                  | 26.7                                 | 6.0   | 0                                 |   |
| Taka-diastase (2 Gm.) | 321                       | 307                                     | $\frac{1}{2}$<br>1<br>1 $\frac{1}{2}$<br>2<br>2 $\frac{1}{2}$<br>3 | 2<br>2<br>7<br>10<br>20<br>27  | 40<br>52<br>56<br>68<br>73<br>85 | 8.8                          | 8.2                                  | 23.0                                 | 35.6  | 35                                | 1.7   |
| 3                     |                           |   |  |                                |                                  |                              |                                      |                                      |   |                                   |   |
| Control               | 73                        | 69                                      | $\frac{1}{2}$  | 2                              | 40                               | 0.66                         | 0.62                                 | 5.2                                  | 11.0  | 0                                 |   |
| Taka-diastase (1 Gm.) | 176                       | 165                                     | 1 $\frac{1}{2}$  | 0                              | 32                               | 4.20                         | 3.9                                  | 12.4                                 | 31.7  | 441                               | 44.1  |

\*Corrected.



*Methods.*—Dogs were anesthetized with pentobarbital, the abdomen was entered through a midline incision, and a segment of intestine from 70 to 90 cm. long was isolated and cannulated at both ends. The segment was washed first with 500 c.c. of water, then 500 c.c. of tenth-normal hydrochloric acid, and then another 500 c.c. of water. From 70 to 100 c.c. of either 3 or 4 per cent potato starch paste was then introduced and permitted to remain for one hour. The unabsorbed fraction of the paste was permitted to drain out, after which the segment was flushed out with 100 c.c. of water, which was collected and combined with the residual paste that had been collected. The segment was then washed with an additional 500 c.c. of water, and the experiment was repeated with 500 mg. of taka-diastase added to the starch paste immediately before introduction. The recovered samples were assayed for amylase, and the reducing sugar and the pH were determined.

TABLE V  
EFFECT OF TAKA-DIASTASE ON STARCH DIGESTION IN THE INTESTINE

| DOG |   | CONC.<br>STARCH<br>PASTE<br>(%) | VOL. STARCH<br>PASTE<br>INTRO-<br>DUCED<br>(C.C.) | VOL.<br>WASH<br>FLUID | TOTAL<br>VOLUME<br>RECOV-<br>ERED | SUGAR<br>RECOV-<br>ERED*<br>(GM.) | STARCH<br>EQUIV-<br>ALENT<br>OF<br>SUGAR | %<br>STARCH<br>DI-<br>GESTED | %<br>ENZYME<br>RECOV-<br>ERED | pH OF<br>RECOV-<br>ERED<br>FLUID |
|-----|---|---------------------------------|---|-----------------------|-----------------------------------|-----------------------------------|--|------------------------------|-------------------------------|----------------------------------|
| 1   | C | 3                               | 100   | 100                   | 190                               | 1.27                              | 0.64                                     | 21.3                         |                               | 6.8                              |
|     | T | 3                               | 100   | 100                   | 180                               | 1.31                              | 0.66                                     | 22.0                         | 95                            | 6.9                              |
| 2   | C | 4                               | 100   | 100                   | 188                               | 1.36                              | 0.68                                     | 17.0                         |                               | 6.8                              |
|     | T | 4                               | 100   | 100                   | 158                               | 1.26                              | 0.63                                     | 16.0                         | 93                            | 6.8                              |
| 3   | C | 4                               | 70  | 100                   | 154                               | 0.99                              | 0.05                                     | 1.9                          |                               | 6.6                              |
|     | T | 4                               | 70  | 100                   | 138                               | 1.04                              | 0.52                                     | 18.5                         | 61                            | 7.2                              |
| 4   | C | 3                               | 100   | 100                   | 184                               | 0.18                              | 0.09                                     | 3.0                          |                               | 7.1                              |
|     | T | 3                               | 100   | 100                   | 150                               | 1.17                              | 0.59                                     | 19.6                         | 65                            | 7.1                              |
| 5   | C | 3                               | 100   | 100                   | 170                               | 0.14                              | 0.07                                     | 2.5                          |                               | 7.1                              |
|     | T | 3                               | 100   | 100                   | 166                               | 1.26                              | 0.63                                     | 21.5                         | 84                            | 7.0                              |
| 6   | C | 3                               | 100   | 100                   | 185                               | 0.15                              | 0.07                                     | 2.5                          |                               | 7.0                              |
|     | T | 3                               | 100   | 100                   | 160                               | 1.22                              | 0.61                                     | 20.5                         | 76                            | 6.8                              |
| 7   | C | 3                               | 100   | 100                   | 170                               | 0.24                              | 0.12                                     | 4.0                          |                               | 7.6                              |
|     | T | 3                               | 100   | 100                   | 150                               | 2.28                              | 1.14                                     | 38.0                         | 72                            | 7.6                              |

\*As glucose.

*Results.*—The results of six such experiments are summarized in Table V. It will be seen that with the exception of the first two experiments, in which pancreatic amylase must have been present in the segment because of inadequate initial washing, starch digestion affected by succus entericus amylase is very limited. It is also seen that taka-diastase is very active in the intestine. Furthermore, the taka-diastase survives one hour in the intestine with only limited loss of activity.

#### EFFECT OF TAKA-DIASTASE ON TOTAL DIGESTION OF STOMACH PLUS SMALL INTESTINE

Gastrointestinal digestion has been studied in one patient with a well-established ileostomy. In such a patient the ileal discharge represents the material which would normally pass into the colon and there be available for bacterial degradation. Such a patient offers an ideal "experimental preparation" for the study of factors affecting the quantity of carbohydrate reaching the colon.

*Methods.*—After hospitalization the patient was placed on a standard weighed diet supplying carbohydrate 177 Gm., protein 66 Gm., and fat 172 Gm. for a period of six days. During the first three-day period he received the unaugmented diet. During the second three-day period he received 2 Gm. of taka-diastase, taken in water, with each meal. The entire output of the ileostomy was collected during the entire experimental period. The material was transferred as soon as excreted to airtight bottles containing sufficient alcohol to make the final concentration 20 per cent. The wet weight, dry weight, starch and nitrogen content of the material were determined for each twenty-four-hour period. Amylase assays at pH 7.2 (pancreatic amylase) and 4.5 (taka-diastase) were carried out during the last two days of taka-diastase therapy.

TABLE VI

EFFECT OF TAKA-DIASTASE ON STARCH AND NITROGEN CONTENT OF MATERIAL DISCHARGED FROM ILEOSTOMY

| DATE  | WET WEIGHT<br>FECES | DRY WEIGHT<br>FECES | TOTAL<br>STARCH | TOTAL<br>NITROGEN | AMYLASE (WILLSTÄTER<br>UNITS 1 C.C.) |        |
|-------|---------------------|---------------------|-----------------|-------------------|--------------------------------------|--------|
|       |                     |                     |                 |                   | pH 4.5                               | pH 7.2 |
| 10/29 | 706                 | 36                  | 2.7             | 1.50              | —                                    | —      |
| 10/30 | 641                 | 44                  | 3.8             | 1.81              | —                                    | —      |
| 10/31 | 878                 | 56                  | 4.8             | 2.12              | —                                    | —      |
| 11/1* | 726                 | 56                  | 6.0             | 1.92              | —                                    | —      |
| 11/2* | 706                 | 48                  | 4.8             | 1.59              | 0.45                                 | 1.8    |
| 11/3* | 746                 | 44                  | 2.1             | 1.70              | 0.55                                 | 1.8    |

\*Taka-diastase (2 Gm.) with each meal.

*Results.*—The results of this study are summarized in Table VI. It will be seen that taka-diastase had no effect either on the nitrogen or on the starch lost in the ileal fluid. This is not remarkable when one notes that even without taka-diastase the amylolytic activity of the collected material was high, as demonstrated by the assay, at pH 7.2 where the effect of the pancreatic amylase would dominate that of the taka-diastase. In other words, the normal pancreas supplies sufficient secretion to maintain potent amylolytic power in the ileal contents. It appears, therefore, that the same fraction of ingested starch that escapes digestion by pancreatic amylase also escapes digestion by taka-diastase.

#### DISCUSSION

It should be clearly understood that the subjects used throughout the experiments on gastric digestion were all healthy young adults, in whom there would be no reason to suspect that enzyme supplementation would be of any practical value. These experiments were undertaken to determine whether conditions in the human stomach are compatible with effective hydrolysis by taka-diastase. It would not be expected that an enzyme preparation would markedly augment digestion in the *normal* stomach. These points are emphasized because, in a sense, this type of experimentation presents the case for enzyme therapy in its least favorable light. Enzyme therapy is a form of substitution therapy and should be reserved for cases in which an enzyme deficiency, whether on the basis of organic disease or poor hygiene, is suspected.

The fact that taka-diastase had no effect on protein digestion is, of course, not unexpected. Taka-diastase possesses only feeble proteolytic activity. The

problem was investigated for two reasons: First, because, theoretically, by liquefying the starch paste adherent to the protein particles it seemed possible that greater surface area would be exposed to the action of pepsin with consequent greater digestion. Second, it will be seen on examining Tables I and II that almost uniformly the pH was higher when taka-diastrase was taken with the meal. When this phenomenon was encountered in the earlier experiments, it was interpreted as possibly being due to greater buffering power resulting from more complete hydrolysis of the protein. As a result, protein as well as starch digestion was investigated in the subsequent experiments. Since the experimental results failed to substantiate the theory, a satisfactory explanation for the pH observations is wanting at the present time. Taka-diastrase has essentially no buffering power, and it seems unlikely that it actively inhibits acid secretion.

The difference between the whole meal and the finely divided meal with respect to the magnitude with which taka-diastrase supplemented "normal" digestion is probably a result of two factors. First, it will be noted (Tables I and II) that with the unground meal the pH of the gastric contents fell more slowly. This permitted the ptyalin to act for a longer period so that, unaided, "maximum" digestion could be effected. Second, it appears likely that the more appetizing unground meal constituted a more potent stimulant for salivary secretion so that larger quantities of ptyalin were available for digestion.

When aided by taka-diastrase, gastric starch digestion in the dog was more complete than it was in man. This is probably due to the fact that in the dog the pH of the gastric contents fell more slowly, thus permitting the taka-diastrase to act for a longer period.

Since in the human studies and in the one dog (No. 2) in which acid secretion approximated that in man only negligible quantities of taka-diastrase were recovered from the stomach in an active form, it must be concluded that when administered in this manner the effect of taka-diastrase is largely confined to the period that it remains in the stomach. However, as has been demonstrated by Ivy, Schmidt, and Beazell,<sup>4</sup> when half the dose of diastrase is administered on a full stomach, a large fraction reaches the intestine in an active form. The studies on the effect of taka-diastrase on digestion in the intestine show that the fraction which escapes inactivation in the stomach continues to be effective.

The failure of the administered diastrase to modify digestion in the patient with an ileostomy could be anticipated. Both the total nitrogen and the total carbohydrate of the material recovered from the ileostomy corresponded with normal values for feces. In other words, in this patient, by the time the terminal ileum had been reached digestion was carried to completion. Thus, supplementation of the digestive ferments could not have an effect.

#### SUMMARY AND CONCLUSIONS

The effect of supplementary amylase on gastric digestion was studied in "normal" human subjects and in dogs with duodenal fistulas.

1. When the tests on the human subjects were conducted with an average mixed meal, it was found that taka-diastrase had no effect on digestion unless

an artificial salivary insufficiency was induced by having the subjects bolt the meal and then refrain from swallowing saliva. Under these latter circumstances taka-diastase augmented gastric starch digestion to a significant extent in 3 of 5 subjects. This meal was recovered from the stomach by voluntary regurgitation on the part of the subjects.

2. With a finely divided mixed meal that could be recovered from the stomach by means of an Ewald tube, taka-diastase effectively augmented starch digestion in all subjects with a 2 Gm. dose, and in 5 of 8 subjects with a 1 Gm. dose. With this meal it was unnecessary to induce a salivary deficiency in order to demonstrate the effect of the enzyme.

3. In the dog the average starch digestion without taka-diastase was 7.58 per cent and with taka-diastase it was 38.9 per cent. The marked difference between the control and taka-diastase experiment is due to the failure of the dog to secrete ptyalin in its saliva. In this respect, results in the dog are indicative of what might be expected in a patient with a marked ptyalin insufficiency.

4. Taka-diastase had no significant effect on gastric protein digestion in the human subjects.

5. Taka-diastase had no significant effect on the rate of emptying of the stomach.

6. In the experiments on the human subjects the quantity of taka-diastase recovered from the stomach in an active form was inconsequential. In the dog, the quantity of taka-diastase evacuated from the stomach in an active form was a function of the rate of secretion of acid by the stomach. This shows that if it is desired to have the enzyme reach the intestine in an active form, it should be administered in water at the completion of the meal, as was shown by Ivy, Schmidt, and Beazell.<sup>4</sup>

The effect of taka-diastase on starch digestion in the intestine was studied by introducing a solution containing a known quantity of starch with and without taka-diastase into an isolated intestinal loop of an anesthetized dog, withdrawing it at the end of an hour, and determining the quantity of reducing sugar that had been formed.

1. Starch digestion without taka-diastase was very limited (maximum 4 per cent). In the presence of taka-diastase from 16 to 38 per cent of the starch was reduced to sugar in the one-hour period.

2. The taka-diastase survived one hour in the intestine with only a limited loss of activity.

Gastrointestinal starch digestion was studied in one patient with a well-established ileostomy. The patient was hospitalized, placed on a standard diet, and the entire discharge from the ileostomy was collected for a period of six days. During the last three days of the experimental period the diet was supplemented with 2 Gm. of taka-diastase taken in water with each meal.

1. Taka-diastase had no effect on the quantity of nitrogen or starch lost in the ileal discharge.

2. The potency of the pancreatic amylase in the ileal fluid was about three times as great as that of the taka-diastase.

3. It appears, therefore, that the fraction of ingested starch that escapes digestion by amylpsin also escapes digestion by taka-diastase.

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## EXPERIMENTAL ASTHMA: TREATMENT WITH HISTAMINASE\*

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## INTRODUCTION

ALTHOUGH most of the major principles of the induction, symptoms, and pathology of experimental asthma in guinea pigs have been investigated, the use of such a model in the evaluation of therapeutic agents has been neglected. The widespread usage of the preparation histaminase or "Torantil"† on a variety of allergic diseases led to such an experimental trial.

The advantages of experimental asthma over anaphylactic shock as a means of studying allergic phenomena are as follows: First, antianaphylaxis does not occur; i.e., animals will react over and over again in a predictable manner with no diminution in sensitivity. This has been repeatedly shown by all investigators in the field and emphasized by Manteufel and Preuner,<sup>1</sup> Kallós and Pagel,<sup>2</sup> and Ratner,<sup>3</sup> and is confirmed below. Thus, the use of the large numbers of animals necessary for any experiment in anaphylaxis is obviated, since an asthmatic animal may be put to repeated tests if a suitable control period is carried out before and after each test. Secondly, the reaction of an animal during an episode of experimentally induced allergic asthma bears a closer resemblance to asthma in man than does anaphylaxis. Furthermore, the degree of severity of the attack is roughly measurable. Finally it produces characteristic pathologic changes in the lungs, simulating the lesions seen in human beings.

Busson<sup>4</sup> in 1911 is said to have been the first to produce asthma by inhalation experiments. Following a number of successful and unsuccessful results

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†"Torantil" was kindly supplied by the Winthrop Chemical Company.

by several workers, Alexander, Beeke, and Holmes<sup>5</sup> in 1926 caused death by inhalation of horse dander extract in guinea pigs previously sensitized either by inhalation or by intraperitoneal injection. They found that strong antigens were necessary, and even then a number of their animals failed to have any reaction. Ratner and co-workers<sup>6</sup> in 1927 produced sensitization, shock, bronchial asthma, and death by nasal inhalation of dry horse dander in guinea pigs. They believed that dry antigen reproduced human conditions better than liquid antigen. Manteufel and Preuner<sup>1</sup> in 1933 called attention to the fact that the spray droplets must be very small in order to produce asthmatic attacks in guinea pigs, and believed that previous failures were due to this fact. They stressed the absence of anti-anaphylaxis and produced asthmatic attacks repeatedly.

Kallós and Pagel<sup>2</sup> published in 1937 the results of a series of investigations on experimental asthma. These authors compared the reactions of guinea pigs subjected to allergic asthma and to inhalation of histamine and of acetylcholine. They described the clinical nature of the attacks, demonstrated the absence of anti-anaphylaxis, showed hereditary transmission of bronchial sensitization, and gave descriptions of the pathology showing certain differences between the three groups of animals. Finally, they demonstrated the ability of atropine, adrenaline, and a calcium preparation to inhibit the attacks.

Recently, Preuner<sup>7</sup> continued his studies and has reported the results of investigations made to learn the effect of the weather on experimental bronchial asthma. By grading the reaction of his animals and recording his results statistically, he concluded that changes in the weather had the effect of increasing the severity of the attack. The effects, however, of change of weather were relatively slight, but his observations established the possibility of measuring the severity of the attack. Ratner's<sup>3</sup> recent review is comprehensive, and he has worked out the time element in sensitization by inhalation of dry antigen.

The question as to whether release of histamine is responsible for anaphylactic shock and allergic phenomena cannot be reviewed here, but some of the more recent evidence is summarized by Laymon and Cumming.<sup>8</sup>

#### HISTAMINASE

The literature concerning histaminase has been accumulating rapidly since the description by Best and McHenry<sup>9</sup> of a substance in animal tissues capable of inactivating histamine when incubated with it for twenty-four hours at 37° C. The substance was observed to have the properties of an enzyme and was found in almost all organs but was most abundant in the intestinal mucosa and the kidney.

Felix<sup>10</sup> found that histaminase had a questionable therapeutic effect on skin diseases and stated that it failed to reduce the gastric acidity of either normal or ulcer patients. Adelsberger<sup>11</sup> and Ertl<sup>12</sup> reported favorable results in such allergic diseases as eczema and hay fever. Foshay and Hagebusch<sup>13</sup> found it of definite value in serum sickness. Roth and Horton<sup>14</sup> have used it effectively in the treatment of cold allergy.

Other reports are not so promising. Laymon and Cumming<sup>8</sup> found histaminase to be beneficial in only certain cases of urticaria. Kile and Rusk<sup>15</sup>

found no improvement in cold allergy after histaminase. A recent article denying the clinical value is that of Miller and Piness,<sup>16</sup> who tried it on a large number of patients with varying allergic disorders. Keeney<sup>17</sup> found no relief of hay fever after its use.

The experimental basis for the use of histaminase has been meager, and its toxicity has not been fully investigated. Karady and Browne<sup>18</sup> reported almost complete prevention of histamine shock in guinea pigs when histaminase was given intravenously fifteen minutes before the administration of a shocking dose of histamine, and a similar result was obtained in inhibiting anaphylactic shock due to egg white in a group of 30 animals. Of 10 animals as controls, 4 died; of the 20 pretreated with histaminase, none died when the homologous antigen was reinjected. The guinea pigs were given 3 units of histaminase intravenously while under ether anesthesia. However, ether anesthesia has been observed to inhibit anaphylactic shock.<sup>19</sup> Corper and Cohn<sup>20</sup> were not able to demonstrate any effect of histaminase on histamine intoxication, tuberculo-anaphylaxis, or tuberculo-allergy.

Inasmuch as the clinical picture presented in animals subjected to experimental allergic asthma is very similar to that seen in human beings with asthma, it was decided to study the influence of histaminase on the experimentally produced disease, where conditions may be more carefully controlled than in patients.

#### ALLERGIC ASTHMA

Following a modification of Kallós and Pagel's<sup>2</sup> methods, young guinea pigs, weighing approximately 10 Gm., were sensitized with 3 intraperitoneal injections at two-day intervals of 0.5 c.c. of fresh egg white diluted 1:5 with distilled water. Ten to fourteen days after the last injection the animals were sprayed with a 10 per cent solution of egg white in normal saline while in a double chamber. The chamber consisted of an inner open glass jar having a capacity of approximately 6 liters placed in a rectangular metal box measuring 77 by 46 by 46 cm. The material was sprayed directly into the inner chamber from which it passed into the larger box. The latter was provided with a small outlet tube. The vaporizer used was a painter's airbrush connected with a compressed air pump. The airbrush was adjusted to give its finest spray, which at the same time was abundant and was such as to vaporize about 5 c.c. of the fluid during a ten-minute period. The animal was sprayed directly and watched from minute to minute.

*Symptoms.*—Most of the animals developed asthmatic symptoms within three minutes after beginning the spray; the attack became maximal after five to ten minutes, continued as long as the spray was continued, even as long as an hour, and subsided in about thirty minutes following withdrawal from the chamber.

A typical reaction was as follows (numbers refer to the grade or degree of reaction used in recording): The first symptoms were motor unrest, ruffling of the fur, sneezes, and scratching the nose (1+). This was followed by a definite increase in the respiratory rate, more frequent sneezes, and onset of cough, usually paroxysmal and violent. The animal often chewed as if salivating (2+).

The cough would continue, although sometimes it subsided, but now in addition to the increased respirations definite dyspnea became manifest; the accessory muscles of respiration came into play, the abdominal muscles were quite active, the alae nasi would dilate, and the expiration was often prolonged. During this stage the animals defecated and urinated (3+). Finally during the most severe and usually maximal phase, respirations were labored, and now slower than in the previous stage, the whole body jerked with inspiration; deeper gasps occurred, coming at irregular intervals. Cyanosis was sometimes perceptible in the ears (4+).

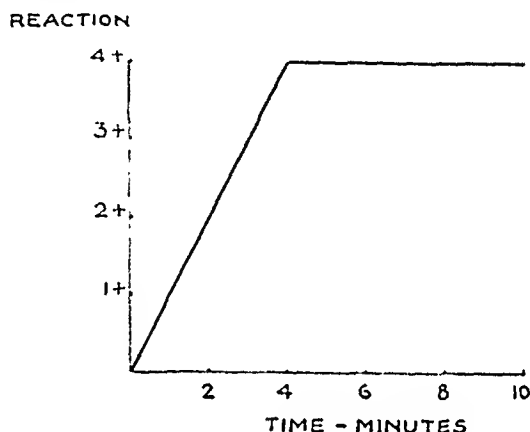


Fig. 1.—Allergic asthma: onset and course of a typical attack.

Some of the animals developed a mild conjunctivitis with lacerimation and edema of the eyelids; this usually persisted after all other symptoms had subsided. Musical whistles and squeaks were audible by stethoscope, and there was some roughening of the respiratory sounds. If the animals were graded by the afore-mentioned numbers, the usual course of events is shown in Fig. 1.

Not all the animals responded alike; in some the onset was more abrupt and in others very slow respirations developed. None of the animals died.

Differential blood counts before and after an attack showed a slight increase of eosinophiles and basophiles within twenty minutes.

Corroborating the work of previous investigators, no antianaphylactic phenomena or desensitization due to repeated attacks was found to occur, even in an animal subjected to 21 attacks of asthma at intervals of from one hour to several days.

*Pathology.*—One animal was sacrificed immediately after its second attack of asthma. Both attacks were maximal, and one month apart. The changes were presumed to be acute, since the animal had had no exposure or symptoms in the interval. Grossly, the lungs filled the entire chest cavity; there was some stringy material in the large bronchi, a few small subpleural hemorrhages, and on section the cut lung surface presented a few areas of reddish discoloration.

Histologically, the most striking change was a wreathing of the bronchi of all sizes with large numbers of eosinophiles, but in addition there were numerous polymorphonuclear cells, and a few lymphocytes and mononuclear cells in an



edematous peribronchial tissue. The capillaries in this area were often plugged with polymorphonuclear cells, and the endothelial cells were swollen and rounded. The bronchial epithelium was swollen and vacuolated; eosinophiles had wandered through the epithelium and were found in moderate numbers in the lumen. The latter was partially filled with mucin, desquamated epithelial cells, red blood cells, and precipitated protein. The bronchioles were often plugged with a similar exudate. Acute emphysema was manifested by stretched and ruptured alveolar walls. Occasional septal accumulations of polymorphonuclear cells, lymphocytes, and eosinophiles were noted. Small areas of atelectasis were also present, with dilated blood-filled capillaries. A few small localized alveolar hemorrhages were present. No eosinophilic pneumonia was seen. Neither true Charcot-Leyden crystals nor Curschmann's spirals were found. Sections of the liver, spleen, heart, and intestine were normal.

#### HISTAMINE ASTHMA

Another group of 7 guinea pigs was subjected to a total of 21 separate sprayings with 0.1 per cent of histamine phosphate at intervals of from one to five days. The symptoms were in a general way similar to those of the sensitized animals sprayed with egg white, but there were notable differences. The onset was usually much more abrupt, severe, and uncontrollable. Some of the animals reacted within three minutes; others not until fifteen minutes had elapsed. Once the reaction began, its progression was very rapid and following severe dyspnea, shock developed with apnea, deep cyanosis and spasms, and the animal fell on its side. The animal appeared moribund and the spray was discontinued. The recovery was equally as rapid as the development of the shock, occurring in three to five minutes. If the animals were sprayed again, the same severe symptoms recurred. The spray was diluted by degrees in the hope of producing a nonshocking reaction resembling allergic asthma, but the animals gave either no reaction or a very severe one. It was almost impossible to produce a definite predictable reaction from day to day as in the allergic asthma. The method thus presented no advantages over parenteral histamine shock, and was deemed unsuited for evaluation of therapeutic agents.

Kallós and Pagel<sup>2</sup> emphasized the differences between histamine and allergic asthma, the most notable difference being the scarcity of eosinophiles in the former. We found that the differences were one of degree rather than of kind, and they were not marked. Grossly, the lungs were again overdistended and showed a few scattered petechiae. A little frothy mucinous material was present in the large bronchi. Histologically, emphysema was again marked, with areas of atelectasis. Peribronchial eosinophilia was present but not quite so intense as in the other animals; the numerous polymorphonuclear cells seen in allergic asthma failed to occur. Hemorrhages were less frequently found. There was, however, edema of certain alveolar septa. Other organs exhibited no changes, but the peribronchial lymph nodes were heavily infiltrated by eosinophiles.

#### HISTAMINASE TREATMENT OF ALLERGIC ASTHMA

Histaminase or "Torantil" is a yellowish white powder supplied in ampoules of 1 histamine-detoxifying unit (1 unit of histaminase is that amount which

will inactivate 1 mg. of histamine at  $37^{\circ}$  C. in twenty-four to forty-eight hours). It is soluble in saline.

In testing the preparation, the first step was the determination of its activity *in vitro*. The drug was weighed out in portions equivalent to 1.0 unit and 0.7 unit. Each quantity was added to 49 c.c. of phosphate buffer at pH 7.35. One milligram of histamine phosphate in 1 c.c. of water was added to each of the two solutions, and to a control solution of buffer alone. The three solutions were saturated with oxygen, sealed and placed in a mechanical shaker in the incubator at  $37^{\circ}$  C.

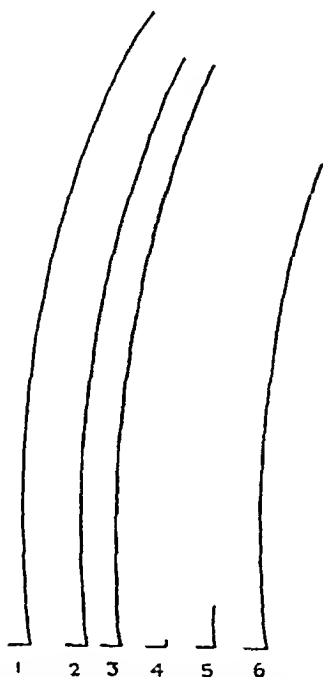


Fig. 2.—Effect of histaminase (Winthrop's Torantil) on histamine contraction of intestinal strip. 1, 0.3 c.c. 1:10,000,000 histamine; 2, 0.3 c.c. 1:10,000,000 histamine; 3, 0.3 c.c. 1:10,000,000 histamine incubated 48 hours at  $37^{\circ}$  C.; 4, 0.3 c.c. 1:10,000,000 histamine from histaminase mixture (1 mg. histamine + 1 unit histaminase) incubated 48 hours at  $37^{\circ}$  C.; 5, same as 4, using 1 mg. histamine + 0.7 unit histaminase; 6, 0.3 c.c. 1:10,000,000 histamine.

The solutions were tested upon the isolated intestinal strip of the guinea pig. At the end of twenty-four hours there was almost complete inactivation in the 1 unit histaminase mixture, and in forty-eight hours it was complete (Fig. 2). It was thus seen that the drug was active *in vitro* according to specifications.

#### METHOD OF TREATMENT

Twelve guinea pigs which had been found to develop allergic asthma of a predictable degree were used. Eight of them were given intravenous control injections of saline while under ether anesthesia. Fifteen minutes later, having recovered from the effects of the anesthesia, they were sprayed in the usual fashion.

After an interval of several days, during which time they were again sprayed and found to develop asthmatic attacks equal in severity to the reactions prior to the saline injection, these 8 animals, and 4 others, were injected

ntravenously with 3 units of histaminase in 2 c.c. of saline. Fifteen minutes later they were sprayed in the usual fashion, and the severity of the reactions was recorded at two-minute intervals for ten minutes. Twenty-four hours following injection of the histaminase, 6 of the group were sprayed again to detect any delayed effect.

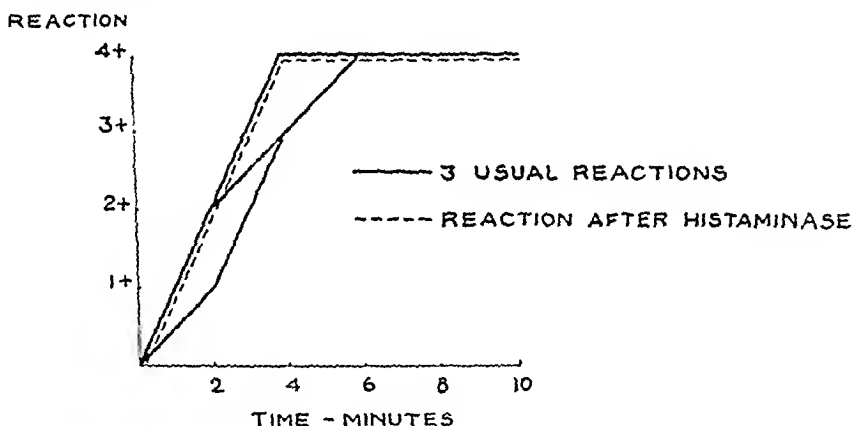


Fig. 3.—Effect of histaminase on onset and course of the typical allergic asthmatic reaction.

#### RESULTS

The result of a typical reaction in one animal, showing its onset and course before and after histaminase, is seen in Fig. 3. The results of all animals is seen in Table I.

TABLE I

EFFECT OF INJECTION OF HISTAMINASE ON ALLERGIC ASTHMA IN GUINEA PIGS, TOGETHER WITH CONTROLS RECEIVING ONLY SALINE

| GUINEA PIG<br>NUMBER | TOTAL NUMBER<br>OF ATTACKS | USUAL<br>REACTION | REACTION<br>AFTER SALINE | REACTION AFTER HISTAMINASE |        |
|----------------------|----------------------------|-------------------|--------------------------|----------------------------|--------|
|                      |                            |                   |                          | 15 MIN.                    | 24 HR. |
| 33                   | 7                          | ++++              | ++++                     | ++++                       | ++++   |
| 34                   | 7                          | ++++              | +++                      | ++++                       | ++++   |
| 35                   | 7                          | ++++              | +++                      | ++++                       | ++++   |
| 36                   | 7                          | ++++              | ++++                     | ++++                       | ++++   |
| 37                   | 7                          | +++               | ++                       | ++                         | ++     |
| 38                   | 7                          | +++               | ++                       | ++                         | ++     |
| 19                   | 9                          | ++++              | +++                      | +++                        |        |
| 22                   | 8                          | ++++              | +++                      | ++                         |        |
| 9                    | 21                         | ++++              |                          | +++                        |        |
| 17                   | 6                          | ++++              |                          | ++++                       |        |
| 23                   | 4                          | ++++              |                          | ++++                       |        |
| 24                   | 4                          | ++++              |                          | ++++                       |        |

It is seen that 5 animals, Nos. 34, 37, 38, 19, and 22, had a lessening in the severity of the attack of one degree after the intravenous injection of the saline solution alone, while 4 animals, Nos. 37, 38, 19, and 9, had a similar diminution in the attack after treatment with histaminase. One animal, No. 8, had a 2+ reaction after histaminase, when its usual reaction was 4+. It would appear from the results with saline alone that either the ether anesthesia or the saline is the cause of this slight decrease, which is itself scarcely significant. There appears to be no latent or delayed effect of histaminase after twenty-four hours,

although the number of animals so tested was relatively small. The onset and course of the allergic reaction appear unchanged by injection of histaminase. In no case was asthma prevented, and in most cases it was the same with or without treatment.



Fig. 4.—Allergic asthma.

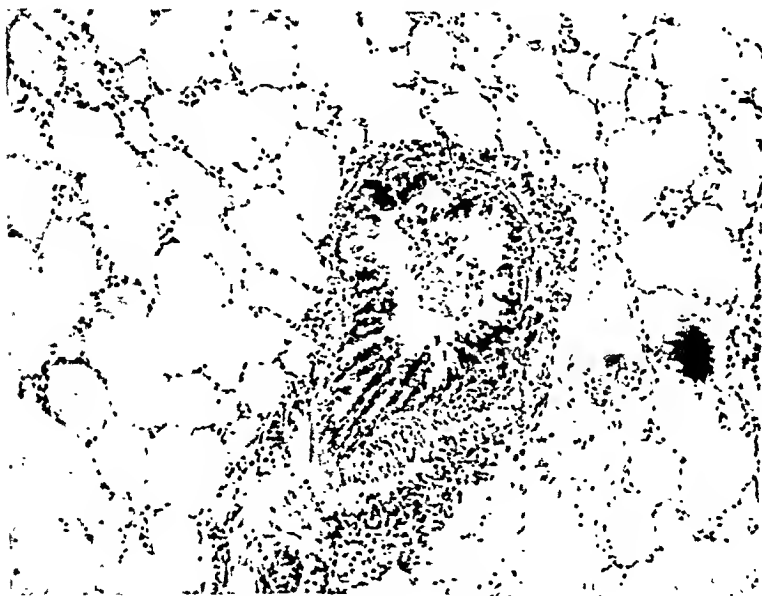


Fig. 5.—Histamine asthma.

The animals injected with histaminase did not seem to recover from the anesthesia as quickly as those injected with saline, but no severe reactions were noted on the first or second injection of the preparation, if it was in the course

of a few days. However, there were 3 animals which received histaminase about three weeks after the first injection; these animals developed severe shock which had all the characteristics of anaphylactic shock, coming on in about five minutes. One died. That this is a true sensitization to the histaminase is not surprising, since histaminase is a protein.

Thus it appears that active histaminase, when administered in amounts said to prevent anaphylaxis, fails to show activity in the prevention of experimental asthma in guinea pigs under the conditions stated.

#### SUMMARY

1. Guinea pigs sensitized intraperitoneally to egg white react to inhaled vaporized antigen in a predictable manner with repeated attacks and show no evidence of desensitization.

2. The symptoms of the reaction resemble human asthma, but the onset and subsidence are perhaps more rapid.

3. The pathologic picture in the lungs is characteristic and is similar in many respects to that seen in bronchial asthma in man.

4. Asthma induced by inhalation of histamine differs from experimental allergic asthma by a more abrupt onset as well as a more rapid disappearance of symptoms. It is, therefore, more difficult to control and measure. Its pathology is similar to allergic asthma but differs in degree.

5. Allergic asthma in guinea pigs is an excellent method for measuring the effect of agents on the reaction.

6. Active histaminase (3 units) given intravenously has no demonstrable effect on allergic asthma in guinea pigs.

7. Sensitization to histaminase may occur with production of anaphylactic shock upon reinjection after a suitable interval.

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## ENTERAL ABSORPTION OF POLLEN ANTIGEN

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WITH the renewed interest in the use of oral pollen therapy the question of absorption has been argued pro and con. The experiments have varied with the investigator and his point of view.

Thommen<sup>1</sup> (1923) cited a patient with ragweed sensitivity to whom ragweed pollen was given orally. He demonstrated that the active antigen was present in the patient's blood and urine, a fact which he concludes is proof that it must have been absorbed. Black,<sup>2</sup> experimenting on himself, ragweed sensitive, found that the ragweed antigen was present in his blood, urine, and feces after the ingestion of the pollen extract. Each of these investigators employed sensitive patients, such as would naturally form the class of person presented to the physician for treatment, and administered daily doses to them.

Such was not the case in the experiments of several other investigators. Bernstein and Kirsner,<sup>3</sup> Bernstein and Feinberg,<sup>4</sup> London,<sup>5</sup> and Zeller<sup>6</sup> administered orally *single doses* of either ragweed pollen extract or whole ragweed pollen to *nonallergic* persons on whom local areas had previously been passively sensitized. They were unable by this method to demonstrate sufficient absorption to cause a reaction in the passively sensitized areas. Of this group of investigators, Zeller comments that by direct skin testing he was unable to demonstrate circulating pollen antigen following *either oral or hypodermic* administration of antigen. He, furthermore, admits that there were certain clinical symptoms following oral therapy which occurred with sufficient frequency that they could be accounted for only on the basis of enteral absorption.

Black, in discussing Zeller's<sup>6</sup> paper, states that he believes the administration of a single dose of pollen extract, even though it is large, will show little or no absorption, since most of it is excreted by the bowel. However, he points out, continued administration over a period of time does give demonstrable amounts in the blood and serum. In pursuance of this phase of the discussion it is interesting to note the report of London.<sup>5</sup> Contrary to the results of Thommen and Black, he was unable to demonstrate the presence of any rag-

weed antigen in sera collected from several of his experimental patients who had taken the pollen orally. Attention is called to the fact that he collected his sera the day after the ingestion of the pollen, whereas Thommen and Black collected theirs on the day of the ingestion. Black notes particularly that after twenty-four hours the concentration of pollen antigen in the sera was greatly reduced. I believe that the important factor is proper timing between the administration of the pollen antigen (either orally or hypodermically) and the sampling of the blood or tissues.

TABLE I

POLLEN CONCENTRATION IN TISSUES OF RABBITS WHICH HAD BEEN GIVEN RAGWEED  
POLLEN ORALLY

| RABBIT | PATIENT   | KIDNEY |        | LIVER  |        | MUSCLE |        | CONTROL<br>EXTRACT<br>(100 UNITS<br>PER C.C.) |
|--------|-----------|--------|--------|--------|--------|--------|--------|---|
|        |           | POLLEN | NORMAL | POLLEN | NORMAL | POLLEN | NORMAL |   |
| 1      | Mrs. S.   | ++++   | +-     | ++     | -      | ++     | -      | ++++  |
|        | Mrs. E.   | ++++   | -      | ++++   | -      | ++++   | -      | ++++  |
|        | Mr. D.    | +++++  | -      |        |        |        |        | +++   |
|        | Miss G.   | +++    | -      |        |        |        |        | ++++  |
|        | Mrs. B.   | ++++   | +-     |        |        |        |        | ++++  |
|        | Mrs. P.   | +-     | +-     | -      | -      | -      | -      | -   |
| 2      | Mrs. S.   |        |        | ++++   | -      |        |        | ++++  |
|        | Miss L.   |        |        | ++++   | -      |        |        | ++++  |
|        | Mrs. W.   |        |        | ++++   | +      |        |        | +++   |
| 3      | Mrs. E.   | ++     | -      | -      | -      |        |        | ++++  |
|        | Mr. D.    | +      | -      |        |        |        |        | +++   |
|        | Mrs. W.   |        |        | +      | +      |        |        | +++   |
| 4      | Mrs. E.   | +++++  | -      | +++    | -      |        |        | ++++  |
|        | Mrs. W.   |        |        | ++     | +      |        |        | +++   |
|        | Miss G.   | ++++   | -      |        |        |        |        | ++++  |
| 5      | Mrs. B.   | ++     | +-     |        |        |        |        | ++++  |
|        | Miss G.   | +      | -      |        |        |        |        | ++++  |
|        | Mrs. Sil. |        |        |        |        | +-     | -      | +++   |
|        | Mrs. E.   |        |        |        |        | ++     | -      | ++++  |
|        | Mrs. S.   |        |        |        |        | ++     | -      | ++++  |
| 6      | Mr. L.    | -      | +-     | ++-    | -      |        |        | +++   |
|        | Mrs. W.   | +-     | +      | ++++   | +-     |        |        | +++   |
| 7      | Mrs. E.   | ++++   | -      | ++     | -      |        |        | +++   |
|        | Mrs. Sp.  | ++     | +-     | ++     | -      |        |        | +++   |
|        | Mrs. B.   | +++++  | +-     | +++++  | +      |        |        | ++++  |
| 9      | Mrs. Sp.  |        |        |        |        | ++     | -      | ++++  |
|        | Mr. Dah.  |        |        |        |        | ++     | +      | ++++  |
|        | Mr. H.    |        |        | ++++   | +-     |        |        | +++   |
|        | Mrs. B.   | +++    | ++-    |        |        |        |        | ++++  |
| 10     | Miss G.   | +      | +      |        |        |        |        | ++++  |
|        | Mrs. Sp.  |        |        |        |        | +++    | ++     | ++++  |
|        | Mr. Dah.  |        |        |        |        | +++    | +      | ++++  |
|        | Mr. H.    |        |        | +++    | +-     |        |        | +++   |
| 15     | Mrs. B.   | +++++  | ++-    |        |        |        |        | ++++  |
|        | Miss G.   | +++    | +      |        |        |        |        | ++++  |
| 15     | Mr. M.    |        |        |        |        | +++    | +-     | ++++  |
| 16     | Mrs. P.   |        |        |        |        | +      | +      | ++  |
|        | Miss H.   | +++    | -      |        |        | +++    | +      | ++++  |

Alperstein<sup>7</sup> (1940), reporting a study of patients sensitive to ragweed who had been placed on oral pollen therapy, comments that the experiments showed (a) that an appreciable amount of pollen is absorbed through the gastrointestinal tract, and (b) that reagin and allergen coexist in the circulating blood at the height of treatment.

Vaughan<sup>s</sup> cites the experience of a physician in connection with a severe attack of asthma suffered by a ragweed-sensitive patient after eating the meat of a pheasant. It was known that the bird's craw had been full of ragweed seed. Accordingly, the physician tested the woman with extract of normal pheasant meat and then with an extract prepared from the meat of a pheasant whose craw was full of ragweed seed. To the former the patient reacted negatively, and to the latter she reacted positively. This suggested the following experiments which I herewith report.

TABLE II

POLLEN CONCENTRATION AT VARYING TIME INTERVALS IN BLOOD SERUM OF RABBITS WHICH HAD BEEN GIVEN POLLEN ORALLY OR HYPDERMICALLY

| RABBIT  | PATIENT   | BLOOD SERUM |            |          |          |          |           |          | CONTROL<br>EXTRACT<br>(100 UNITS<br>PER C.C.) |
|---|-----------|-------------|------------|----------|----------|----------|-----------|----------|---|
|   |           | 0<br>HR.    | 15<br>MIN. | ½<br>HR. | 1<br>HR. | 2<br>HR. | 2½<br>HR. | 3<br>HR. |   |
| 6<br>Ragweed pollen<br>orally                                       | Mrs. Sil. | +           | -          |          |          |          |           | +++      | +++   |
|   | Mrs. E.   | +           | -          |          |          |          |           | +++      | +++   |
|   | Mrs. S.   | -           |            |          |          |          |           | ++       | +++   |
|   | Mrs. W.   | +           |            |          |          |          |           | +++      | +++   |
| 7<br>Ragweed pollen<br>orally                                       | Mrs. E.   | ++          |            |          |          |          | +++       |          | +++   |
|   | Mrs. Sp.  | -           |            |          |          |          | +         |          | +++   |
|   | Mrs. B.   | ++          |            |          |          |          | +++       |          | +++   |
| 8<br>Ragweed pollen<br>orally                                       | Mrs. Br.  | -           |            | - *      |          |          |           |          | +++   |
|   | Mrs. M.   | -           |            | ++       |          |          |           |          | +++   |
| 11<br>Ragweed pollen<br>orally                                      | Mrs. Br.  | -           |            | - *      |          |          |           |          | +++   |
|   | Mrs. M.   | -           |            | ++       |          |          |           |          | +++   |
| 12<br>Ragweed pollen<br>orally                                      | Mrs. G.   | +-          | ++         |          | ++       | +        |           | -        | +++   |
|   | Mr. F.    | -           | +          |          | +        | +        |           | -        | ++  |
|   | Mr. G.    | ?           | ?          |          |          | ?        |           |          | +-  |
|   | Mr. R.    | ?           | +          |          |          |          |           |          | ++  |
| 13<br>60,000 units rag-<br>weed pollen<br>given hypoder-<br>mically | Miss G.   | ?           | ?          |          | +-       |          | +         |          | +++   |
|   | Mrs. B.   | -           | -          |          |          |          | +         |          | ++  |
|   | Miss Sw.  | +           | +++        |          | ++++     |          | +++       |          | +++   |
| 14<br>Ragweed pollen<br>orally                                      | Mrs. Ber. | +           |            | +++      |          |          |           |          | +++   |
| 16<br>Ragweed pollen<br>orally                                      | Mrs. B.   | +-          |            |          | ++       |          |           |          | ++  |
| 17<br>Timothy pollen<br>orally                                      | Mrs. K.   | +-          |            |          |          | ++       |           |          | +++   |
|   | Mr. B.    | +           |            |          |          | ++       |           |          | +++   |

\*Twenty-four hours later the "½ hour serum" gave an inflammatory reaction 3 inches in diameter.

#### EXPERIMENTATION

Rabbits (either small or large) were given defatted ragweed pollen orally. Approximately two to two and one-half hours later they were killed. The kidneys, liver, and a piece of leg muscle were removed and quickly rinsed with tap water. Each was then ground and extracted with a solution consisting of 5 per cent glycerin in normal saline, 1 c.c. of extracting fluid being used for each gram of tissue. The tissues were allowed to extract in the icebox for



twenty-four to forty-eight hours, at the end of which time they were passed through a Seitz filter for sterility. Of the 19 rabbits used in the experiment, two were normal and furnished tissues for the control. To each of the other 17 1 Gm. of pollen was given orally.

Each of the extracts, together with a normal tissue extract control and a ragweed extract control, was tested on several ragweed-sensitive persons. Likewise, some of them were tested on a normal nonallergic person. All skin tests were done by intradermal method.

The results are given in Table I. A summary of the results showed that in ragweed-sensitive patients the control tissue extract gave a *negative to a two-plus wheal without pseudopodia*, while the extracts from the tissue of the rabbits which had been given the ragweed pollen orally, gave, in most cases, a *two-plus to a four-plus reaction with pseudopodia*. The control ragweed extract gave on the average a four-plus wheal with pseudopodia. These results were so definite that there can be no question that some of the pollen antigen was absorbed.

Blood was collected from some of the rabbits just before the oral administration of the pollen and at varying time periods afterward. The sera from these specimens were separated and then tested intradermally on pollen-sensitive patients. The results are shown in Table II and indicate that the active pollen antigen appears in appreciable quantities in the blood fifteen minutes after the oral administration of the pollen, but that by two and one-half hours its concentration is decreasing. For comparison one rabbit was given subcutaneously 60,000 Noon units of ragweed pollen extract while another was given orally 1 Gm. of timothy pollen. These results also are shown in Table II.

#### DISCUSSION

The evidence to date indicates overwhelmingly that the active pollen antigen is absorbed in appreciable amounts when given by the enteral method. This evidence consists of (1) reports in the literature of 1,409 hay fever cases which had been treated by oral pollen therapy with definite improvement in at least 944 cases; (2) demonstration that the active antigen can be found in the blood and tissue of the rabbit and in the blood and urine of man after oral administration of pollen; and (3) positive reactions following enteral ingestion of pollen. These reactions can be interpreted only on the basis of absorption. Even the opponents of the oral method speak of reactions, and it is obviously inconsistent to cite the occurrence of these while maintaining that no absorption takes place.

The only negative evidence is that enteral absorption of pollen antigen is insufficient to produce a reaction in the passively sensitized skin of nonallergic persons, whereas such a reaction is produced by hypodermic injection. However, if a tourniquet is placed above the point of injection, thus slowing down the rate of absorption of pollen extract, the same negative result is obtained in the latter case. Hence, it is probable that the rate of absorption of ingested pollen is too slow to induce the local speed of reaction necessary in the passive transfer tests. This is further evidence of the safety of oral pollen therapy.

## SUMMARY

Experimental evidence is presented which shows that after the oral ingestion of pollen by rabbits the active pollen antigen appears in the blood and tissues, kidney, liver, and muscles.

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## AGE INCIDENCE OF POSITIVE TUBERCULIN REACTIONS (MANTOUX)\*

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WITH A STATISTICAL ANALYSIS BY  
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THE intracutaneous test with old tuberculin Koch (Mantoux test) has been used by many workers since 1909 to determine the incidence and the distribution of tuberculous infection. It has been shown that in a given community the incidence of positive cutaneous reactions to tuberculin depends on the size of the population, whether urban or rural, the incidence of active tuberculous disease, and the living conditions, particularly with respect to hygienic standards and the degree of overcrowding.<sup>1</sup>

It has also been shown that the percentage incidence of positive reactions to tuberculin and the age at which the tuberculin test becomes positive may be decidedly different for different cities, and for similar classes of people.<sup>2</sup> In Vienna<sup>3</sup> in 1909 the incidence of positive tuberculin reactions, in individuals clinically free from active tuberculosis, was found to be very high among the poorer classes; one-half the children showed a positive reaction at 5 years of age; about 90 per cent at 10 years, and 94 per cent at puberty. In New York City the incidence was found to be considerably lower during the years 1921 to 1928 by Smith,<sup>4</sup> and in 1934 in a survey made by one of us (F. P.).<sup>5</sup> Table I illustrates the striking difference in age incidence of positive tuberculin re-

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actions in comparable sections of the populations of Vienna and New York City. The somewhat higher incidence in Smith's series as compared with the group studied by one of us (F. P.) may be explained by the fact that Smith's group included cases of active tuberculosis.

TABLE I  
COMPARISON OF AGE INCIDENCE OF POSITIVE TUBERCULIN REACTIONS

| AGE   | VIENNA<br>1909 (%) | NEW YORK CITY<br>1921-1928 (%) | NEW YORK CITY<br>1934 (%) |
|-------|--------------------|--------------------------------|---------------------------|
| 5     | 50                 | 14.7                           | 9.5                       |
| 10    | 90                 | 37                             | 29.1                      |
| 12-13 | 94                 | 45.5                           | 35.2                      |

The incidence of positive tuberculin reactions may vary with changing conditions. In the same community the lapse of a decade or a few years may influence the statistics considerably.<sup>6</sup> Chadwick and Zacks<sup>7</sup> reported a reduction of 23 per cent in positive reactions among children of the same age groups between the years 1917 and 1926, coincidental with an intensive antituberculosis campaign. It is important, therefore, when interpreting results to take into consideration the year or the period during which a survey is made.

#### EXPERIMENTAL

We should like to report the results of our studies in the age incidence of positive tuberculin reactions carried out in a group of 362 persons, ranging in age from a few months to 80 years.

The persons in whom the tests were made were patients treated for various dermatoses at the Skin and Cancer Unit during the year 1939. The clientele of this clinic consists predominantly of white urbanites from the more crowded districts of New York City, whose economic status may be rated as poor or lower middle class. Patients suffering from any form of skin tuberculosis were excluded from the study.

Old tuberculin Koeh, obtained from the Department of Health of the City of New York, was used, and the Mantoux technique was employed. The initial test dose was 0.02 mg. of O. T. (0.1 c.c. of 1:5,000 dilution). The patients were retested with 0.1 mg. of O. T. (0.1 c.c. of 1:1,000 dilution) and 1.0 mg. O. T. (0.1 c.c. of 1:100 dilution) if the first injections failed to elicit a reaction.

The test site was examined forty-eight hours later, and the reaction was graded as one plus to four plus, as recommended by the National Tuberculosis Association.<sup>8</sup> The patients were then classed as belonging to four different groups, depending on the intensity of the reaction and the concentration required to elicit a reaction:

- I. Strongly positive—if a three-plus to four-plus reaction was elicited with 0.02 mg. O. T. Koeh.
- II. Moderately strong positive—if a one-plus to two-plus reaction was elicited with 0.02 mg. O. T.
- III. Weakly positive—if either 0.1 mg. or 1.0 mg. O. T. was required to elicit a reaction.
- IV. Negative—if the patient failed to react to 1.0 mg. O. T.

## RESULTS

In the age groups of the first two decades there was a gradual rise in the incidence of positive reactions to 33.3 per cent. Between the twentieth and twenty-ninth years the rise in the incidence of positive reactions was sharp, from 33.3 per cent to 90.9 per cent. After the thirtieth year the positive incidence remained fairly constant, varying between 90 per cent and 98 per cent.

TABLE II  
FREQUENCIES OF POSITIVE TUBERCULIN REACTIONS AT VARIOUS AGES IN A  
RANDOM SERIES OF 362 PERSONS

| AGES   | NUMBER OF<br>PERSONS TESTED | NUMBER OF<br>NEGATIVE<br>REACTIONS | POSITIVE REACTIONS |            |
|--------|-----------------------------|------------------------------------|--------------------|------------|
|        |                             |                                    | NUMBER             | PER CENT*  |
| 0-4    | 26                          | 26                                 | 0                  | 0          |
| 5-9    | 20                          | 19                                 | 1                  | 5.0 ± 3.2  |
| 10-14  | 25                          | 18                                 | 7                  | 28.0 ± 6.0 |
| 15-19  | 33                          | 22                                 | 11                 | 33.3 ± 6.0 |
| 20-24  | 40                          | 17                                 | 23                 | 57.5 ± 5.1 |
| 25-29  | 44                          | 4                                  | 40                 | 90.9 ± 2.9 |
| 30-39  | 73                          | 4                                  | 69                 | 94.5 ± 1.8 |
| 40-49  | 50                          | 1                                  | 49                 | 98.0 ± 1.3 |
| 50-59  | 32                          | 1                                  | 31                 | 96.9 ± 2.0 |
| 60-80  | 19                          | 2                                  | 17                 | 89.5 ± 5.7 |
| Totals | 362                         | 114                                | 248                |            |

\*The figure following the ± sign represents the probable error of the observed frequency.

In Table II are listed the frequencies of positive tuberculin reactions among 362 persons at various ages, and in Fig. 1 these results are presented graphically. The observed differences in incidence of positive tuberculin reactions at varying ages are statistically significant. The probable error of an observed frequency is calculated from the formula:

$$\text{Probable Error} = 0.6745 \sqrt{\frac{p(1-p)}{N}}$$

where  $p$  is the observed frequency and  $N$  is the number of persons in the sample tested. This formula was applied to the frequencies obtained for positive tuberculin reactions in various age groups, the results being listed in the last column of Table I. As an example, the calculation for age group 25 to 29 is given:

$$\text{P.E.} = 0.6745 \sqrt{\frac{(0.909)(0.091)}{44}} = 0.029, \text{ or } 2.9 \text{ per cent.}$$

The formula is said to be accurate only for samples containing 30 or more persons, but for the sake of uniformity it was also used for age groups 0 to 4, 5 to 9, 10 to 14, and 60 to 80, each of which contained less than 30 persons. However, the error in doing this is not great, since the smallest group contained 19 persons.

The frequencies of positive tuberculin reactions in two different age groups can be compared by determining the differences between the two frequencies and then comparing this difference with its probable error. The probable error of a difference between two independent frequencies  $p_1$  and  $p_2$  is calculated by using the formula:

$$\text{P.E.}_{\text{Diff.}} = \sqrt{(\text{P.E.}_{p_1})^2 + (\text{P.E.}_{p_2})^2}$$

The odds that a particular difference is significant, that is, not purely accidental, is determined with the aid of Table III. The usual convention is to consider as significant any difference which is more than three times its probable error.

TABLE III

| SIZE OF DIFFERENCE IN RELATION<br>TO ITS PROBABLE ERROR | ODDS THAT DIFFERENCE<br>IS SIGNIFICANT |      |
|---|--|------|
| $\pm 1$ P. E.   | 1                                      | to 1 |
| $\pm 2$ P. E.   | 3.6                                    | to 1 |
| $\pm 3$ P. E.   | 21                                     | to 1 |
| $\pm 4$ P. E.   | 140                                    | to 1 |
| $\pm 5$ P. E.   | 1,300                                  | to 1 |
| $\pm 6$ P. E.   | 19,200                                 | to 1 |
| $\pm 7$ P. E.   | 427,000                                | to 1 |
| $\pm 8$ P. E.   | 14,703,000                             | to 1 |
| $\pm 9$ P. E.   | 730,000,000                            | to 1 |
| $\pm 10$ P. E.  | 65,000,000,000                         | to 1 |

As an example, let us compare the incidence of positive tuberculin reactions in age groups 10 to 14 and 20 to 24. Here  $p_1 = 28.0$  per cent and  $P.E.p_1 = 6.0$  per cent;  $p_2 = 57.5$  per cent and  $P.E.p_2 = 5.1$  per cent.

Therefore,  $p_2 - p_1 = 29.5$  per cent and  $P.E. (p_2 - p_1) = \sqrt{(0.06)^2 - (0.051)^2} = 0.081$ , or 8.1 per cent.

Since  $p_2 - p_1$  is more than three times its probable error, this difference is probably significant. How increasing the size of the samples helps to demonstrate differences among the age groups is illustrated by pooling together the results for ages 0 to 24 and comparing these with the observations made on persons more than 25 years old. Among 144 persons under 25 years old the incidence of positive tuberculin reactions was  $29.2 \pm 2.9$  per cent; among 218 persons older than 25 years it was  $94.0 \pm 1.1$  per cent. Accordingly, the difference between these two groups was  $64.8 \pm 3.1$  per cent, so that this difference was more than twenty times its probable error!

#### DISCUSSION AND SUMMARY

We are of the opinion that the sharp rise in the incidence of positive tuberculin reactions during the third decade, from 33.3 per cent to 90.9 per cent, may be of clinical and epidemiologic significance.

It has generally been held that in urban communities the majority of persons are infected with tuberculosis by the time adolescence is reached. Our findings, and the recent studies of others, indicate that this may not be so. Smith<sup>4</sup> reported a positive incidence of 45 per cent in persons from 10 to 15 years old; Drolet,<sup>9</sup> 29 per cent; Dickey and Seitz,<sup>10</sup> 37 per cent; Aronson and Nicholas,<sup>11</sup> 31.8 per cent. Soper and Amberson<sup>12</sup> state, "in this country at large, roughly speaking, 50 per cent of the white population under the age of twenty does not react to tuberculin."

If the tuberculin reaction is used as the criterion of past or present tuberculous infection, our results indicate that under present conditions in New York City the majority of persons are not infected until adult life. Long<sup>13</sup> has shown, however, that the tuberculin test may fail to detect all healed pri-

mary tuberculosis because of the waning of cutaneous sensitivity that occurs with healing. One cannot assume, therefore, that the rise of positive tuberculin reactions during the third decade is due entirely to initial infection with tubercle bacilli. Superinfection or reinfection of sufficient magnitude to heighten tuberculin sensitivity, spontaneous fluctuation in sensitivity, or increase of sensitivity due to maturation or other factors, must also be considered as the possible *modus operandi* in some cases.<sup>o</sup>

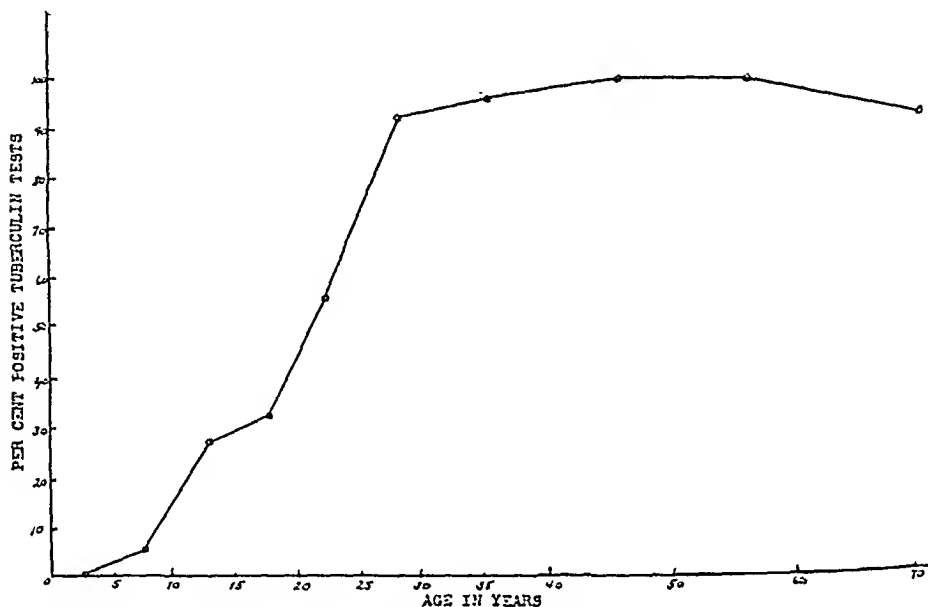


Fig. 1.—Incidence of positive tuberculin reactions at various ages based on a study of a random series of 362 persons.

The report of the New York State Department of Health (Division of Tuberculosis) in the annual report of 1937<sup>14</sup> confirms the clinical significance of our findings. The largest number of new cases of active tuberculous disease of all types was reported during the third decade. This finding is in striking conformity with the present demonstration of sharp increase in incidence of cutaneous tuberculin sensitivity in precisely this age group.

The limitations of the tuberculin test as a sole means of case finding have been pointed out by Plunkett,<sup>15</sup> and we are aware that the tuberculin test is of questionable value as a means of establishing an epidemiologic index of tuberculous infections.<sup>16</sup> Study of age incidence of positive tuberculin reactions may serve, however, to indicate the age groups in which an intensive search for new cases should be made.

Under the present conditions in New York City the results of quantitative tuberculin skin tests coincide with the clinical statistics, showing that for the appearance of active tuberculous disease, the third decade appears to be the most vulnerable age.

<sup>o</sup>There are many possible explanations for the 9 per cent drop in positive reactions in the seventh and eighth decades, but these cannot be entered into here.

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## THE QUESTION OF PROTECTION AGAINST HISTAMINE AND ANAPHYLACTIC SHOCK IN GUINEA PIGS BY HISTAMINASE\*

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THE *in vitro* inactivation of histamine by histaminase preparations is a slow process even under optimal conditions, as shown by Best and McHenry.<sup>1, 2</sup> However, Karady and Browne<sup>3</sup> found that histaminase preparations injected into etherized guinea pigs exerted a protective effect against lethal doses of histamine, and also against anaphylactic shock. They concluded that histaminase acts more rapidly *in vivo* than *in vitro*, and that their observations afforded experimental support for the reported therapeutic effectiveness of histaminase preparations against allergic responses in man.

Haag and Latz<sup>4</sup> also performed experiments which they interpreted as indicating a protective effect of histaminase against anaphylactic shock. This interpretation is not well supported by their experimental results, since, when normal guinea pigs were used, 12 showed greater symptoms when shocked twelve hours after "protection" with a histaminase preparation than did the 14 similarly sensitized and shocked controls which were not given a protective injection. Haag and Latz ignored these animals and drew their conclusions

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\*From the Laboratories of Dr. George Piness and Dr. Hyman Miller, Los Angeles. Received for publication, December 2, 1940.

from the results of a similar test done with 29 animals which had survived a (negative) trial of the desensitizing action of multiple small doses of histaminase. Alternative explanations of the mechanism of the protective action suggested by this experiment would appear to be possible, in view of the facts that histaminase preparations contain antigenic hog proteins and that the "protective" injections were immediately followed by slight but definite symptoms suggestive of anaphylactic shock.

Our own trials of histaminase preparations in man for the treatment of allergic conditions<sup>3</sup> and for the control of histamine or allergic skin reactions<sup>6</sup> did not give any definite indication of in vivo activity or of therapeutic effect. It was, therefore, thought desirable to repeat the experiments reported by Karady and Browne.

With one exception, the observations of Karady and Browne did not include any control animals handled in the same way as those treated with histaminase (etherization and intrajugular injection). This exception consisted of three guinea pigs given heat-inactivated histaminase followed by histamine, and it was found that these died more quickly than the controls given histamine alone. Not only were these animals too few in number to serve as convincing procedure controls, but there is a possibility that heating may have produced toxic properties in the histaminase solution.

In order to simplify our technique, and to conserve our limited supply of histaminase, we compared the protective effect of histaminase solutions with control saline solutions, both solutions being injected intracardially into unanesthetized animals.

#### EXPERIMENTAL

##### A. Solutions and Procedures.

*Saline.* An 0.85 per cent sodium chloride solution containing 1:1,000,000 of phenylmercuric acetate as preservative.

*Buffered Saline.* A solution of sodium chloride, disodium phosphate, and monopotassium phosphate, having a pH 7.4, a ratio of sodium to potassium ion concentrations of 40:1, and an ionic strength equal to that of 0.85 per cent sodium chloride. Same preservative.

*Histaminase.\** "Parenteral histaminase," a soluble powder, received in sealed glass ampoules. For Experiment I the contents of 42 one-unit ampoules were dissolved in 28 ml. of buffered saline about two hours before use. For Experiment II, Part 1, five one-unit ampoules and 20 two-unit ampoules were dissolved in 30 ml. of saline about two hours before use. For Experiment II, Part 2, 20 ten-unit ampoules of a special experimental lot were dissolved in 27 ml. of buffered saline three hours before use. After two guinea pigs had been killed immediately (with pulmonary and pleural hemorrhages) by intracardial injection of the last solution at the usual dose of 8 ml. per kilogram, it was diluted 1:5 with buffered saline in order to bring it to the same strength (in labeled units) as the other histaminase solutions.

\*Histaminase was supplied through the courtesy of the Winthrop Chemical Co. and Dr. J. B. Rice, its Director of Medical Research. The one-unit ampoules were designated "T-360-1," and the two-unit ampoules, "T-360." Samples of these were returned to the manufacturers for re-assay just before Experiment II, Part 1, was done. They reported that the "T-360-1" had dropped in potency to about 60 per cent of the labeled value, while the "T-360" retained its original potency.



**Histamine.** A solution containing 10 mg. of histamine dihydrochloride (Hoffman-LaRoche) per milliliter in buffered saline was freshly prepared for each experiment in which it was used.

**Egg Albumen.** The stock from which solutions were made is a four times crystallized preparation,<sup>7</sup> consisting of the washed crystal mush, pressed between blotters to remove most of the wash liquor, dried rapidly in a current of warm air, and stored in the cold. Nitrogen analyses showed that about 1.5 per cent of the egg albumen was denatured by drying, and that the total content of egg albumen was 64.2 per cent, while the content of ammonium sulfate was 31.7 per cent. For sensitization 46.7 mg. of the dry material was dissolved in 30 ml. of buffered saline (1 mg. of egg albumen per milliliter), and for shocking, 4.67 Gm. were dissolved in 30 ml. of buffered saline (100 mg. of egg albumen per milliliter).

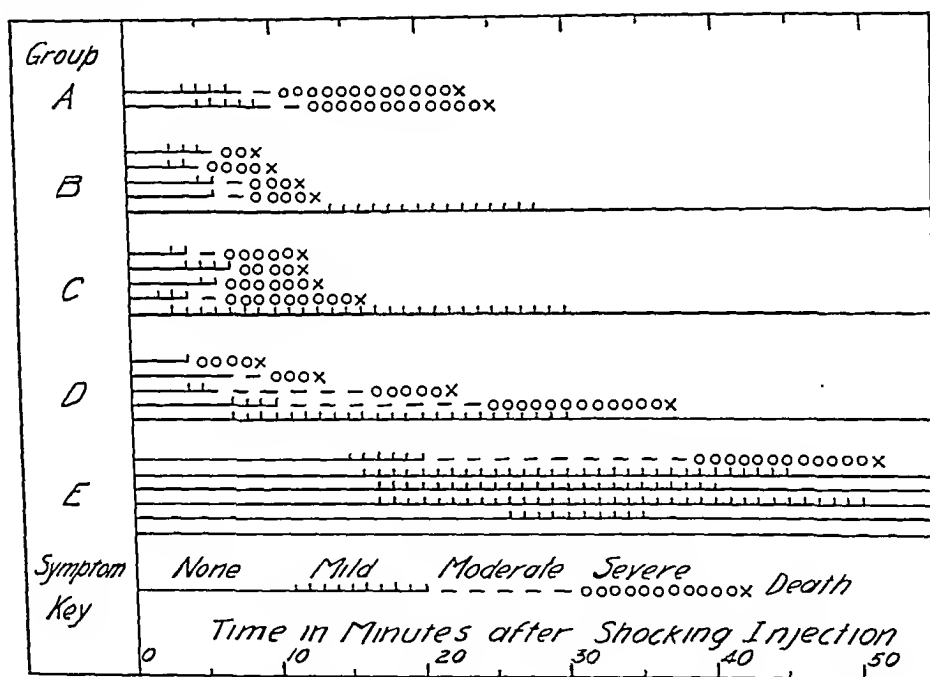


Fig. 1.—Experiment I—Histamine shock.

The first guinea pig in group D was depressed and uncertain on his feet between four and six minutes after the first injection.

\*This histaminase solution contained, according to re-assay, approximately 0.9 units per milliliter, instead of the intended 1.5 units per milliliter.

**Doses and Injection Technique.** Because our guinea pigs differed in weight from those used by Karady and Browne, it seemed desirable to give the histaminase and histamine in proportion to weight. Doses of 8 ml. per kilogram of histaminase solution, and 2.5 ml. per kilogram (25 mg. per kilogram) of histamine dihydrochloride solution were selected as a fair approximation to the average dose used by them. In the first experiment, however, two guinea pigs

were given 15 mg. of histamine dihydrochloride per kilogram (group A), and one group of animals (group C) was given 2 ml. of histaminase solution without regard to weight.

Histamine solutions were injected intraperitoneally. Histaminase and control saline solutions were given intracardially, using a  $1\frac{1}{4}$  inch, No. 22 gauge needle, and entering the chest through the diaphragm, inserting the needle along the left side of the xiphoid process. The solutions were injected slowly, testing for needle placement by drawing a drop or two of blood up into the syringe at the beginning and end of the injection. Judging by the color of the blood, almost all the injections were made into the left side of the heart. The animals withstood the injections very well, with the exception of the first guinea pig of Experiment I, group D. The histaminase or control saline solution was always administered fifteen minutes before the shocking injection.

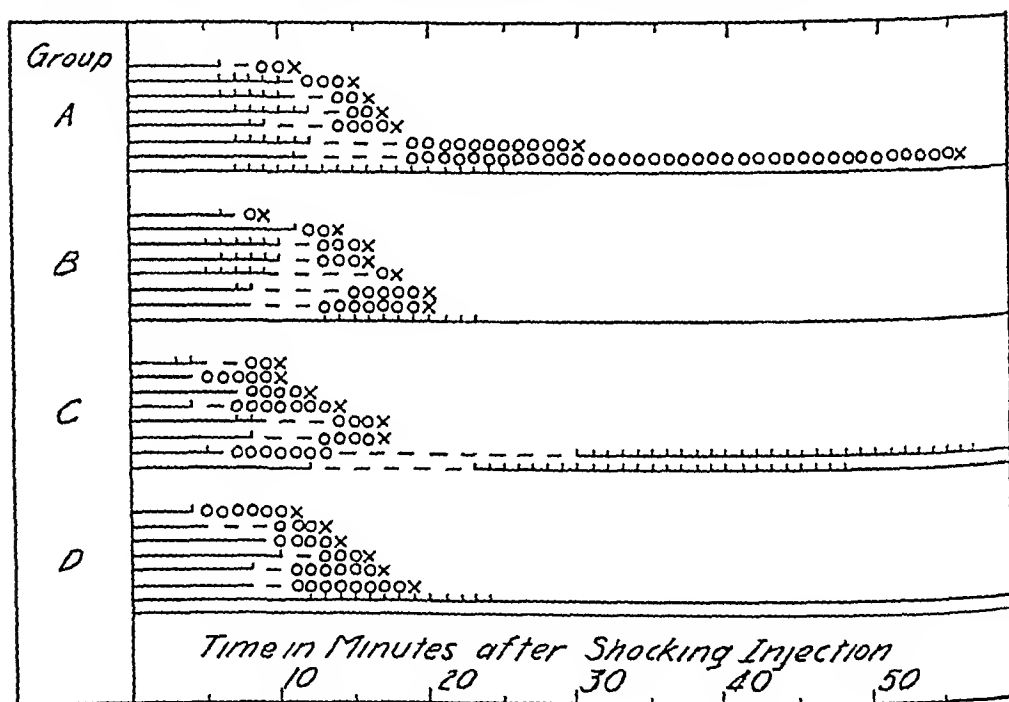


Fig. 2.—Experiment II, Part 1—Histamine shock.

| Group | "Protecting" Injection<br>(Intracardial) | Shocking Injection<br>(Intraperitoneal) |
|-------|--|---|
| A     | None                                     | 25 mg./kg. histamine dihydrochloride    |
| B     | 8 ml./kg. saline                         | 25 mg./kg. histamine dihydrochloride    |
| C     | 8 ml./kg. histaminase*                   | 25 mg./kg. histamine dihydrochloride    |
| D     | 8 ml./kg. buffered saline                | 25 mg./kg. histamine dihydrochloride    |

(For symptom key, see Fig. 1.)

\*This histaminase solution contained, according to re-assay, approximately 0.9 unit per milliliter, instead of the intended 1.5 units per milliliter.

The sensitizing and shocking doses of egg albumen were given without regard to animal weight, 1 ml. of the respective solution being administered intraperitoneally. The sensitizing dose was given two days after the animals were received, and the shocking dose was administered six weeks later. To assure ourselves that the ammonium sulfate content (49.4 mg. per milliliter) of the shocking solution was not high enough to be toxic, and that the solution was otherwise free of primary toxicity, three nonsensitized guinea pigs (group

E, consisting of three of the six survivors of Experiment II, Part 1, which was done fourteen days previously) were given the shocking dose of egg albumen solution. They showed no signs of difficulty or discomfort at any time.

*Animals.* All guinea pigs were obtained from a single source and were uniform in age. Those for Experiment I weighed from 480 to 650 Gm. Those for Experiment II, both parts, all obtained at one time, were all white, weighed from 200 to 250 Gm. when received, from 340 to 480 Gm. when Part 1 (histamine shock) was done four weeks later, and from 400 to 510 Gm. when Part 2 (anaphylactic shock) was completed. The animals were not selected for sex. A record of sex was kept for Experiment II, but no differences were seen. The guinea pigs were fed plenty of greens, supplemented by rabbit pellets, and water was available to them at all times.

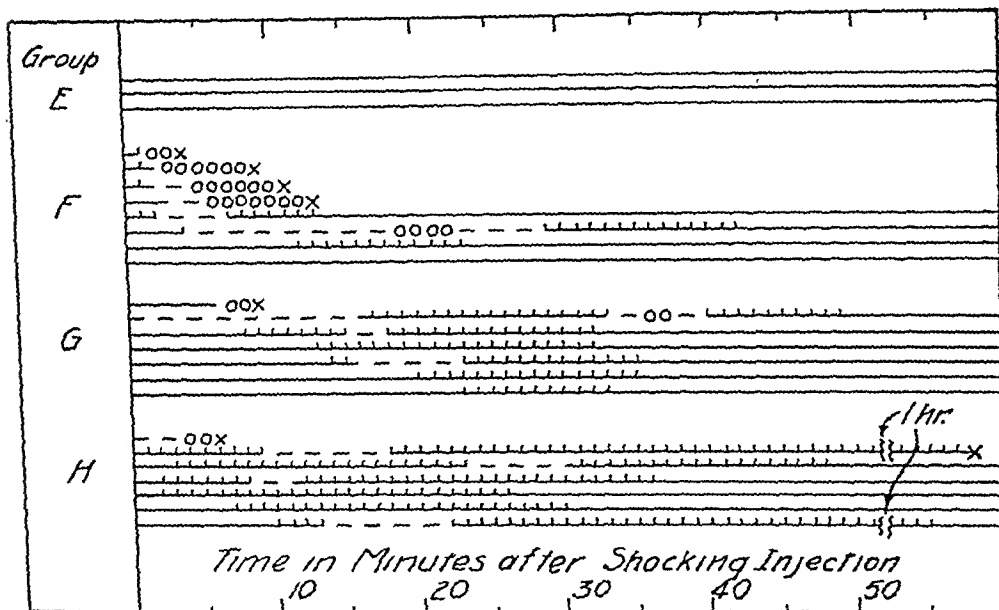


Fig. 3.—Experiment II, Part 2.—Anaphylactic shock.

| Group | "Protecting" Injection<br>(Intracardial) | Shocking Injection<br>(Intraperitoneal) |
|-------|--|---|
| E     | (Not sensitized, see text)               | 100 mg. egg albumen                     |
| F     | None                                     | 100 mg. egg albumen                     |
| G     | 8 ml./kg. histaminase*                   | 100 mg. egg albumen                     |
| H     | 8 ml./kg. buffered saline                | 100 mg. egg albumen                     |

(For symptom key, see Fig. 1.)

\*This histaminase solution contained 1.5 units per milliliter.

### B. Experiments.

The results of the experiments are shown in the diagrams: Fig. 1 for Experiment I, Fig. 2 for Part 1 of Experiment II, and Fig. 3 for Part 2 of Experiment II. Each line represents a guinea pig, and the key to the symbols is shown in Fig. 1. The symptoms were classified as mild, moderate, and severe, in the order of their usual development, as follows:

#### Histamine shock symptoms:

Mild: depression, lowered respiratory rate, scratching.

Moderate: respiratory difficulty, coughing, gasping.

Severe: collapse, convulsions.

### Anaphylactic shock symptoms:

Mild: restlessness, ruffled fur, sneezing, scratching, loss of sphincter control.

Moderate: irregular respiration, jumping.

Severe: rearing, gasping, collapse, convulsions.

(Depression is the principal symptom after dyspnea has disappeared, when animals show signs of recovering.)

All the animals which have been used by us in this type of experiment are included in the results.

### DISCUSSION AND CONCLUSIONS

A slight prolongation of survival time after histamine by histaminase solutions occurred in both C and D of Experiment I, and a marked prolongation in anaphylactic shock in Experiment II, Part 2. To this extent our data agree with the findings of Karady and Browne.<sup>3</sup> However, in no experiment was there any definite indication that the histaminase solution had greater efficacy than an equivalent volume of the saline solution used to dissolve the histaminase; and in Experiment I more of the saline solution group of animals survived than of the histaminase treated groups. However, none of the differences between any two groups of animals is statistically significant. Therefore, the only conclusion which may safely be drawn from the experiments is that Karady and Browne's statement that histaminase protects animals against histamine and anaphylactic shock is not supported either by the evidence they offered or by our findings. It should be pointed out that this cannot be taken as proof that histaminase preparations altogether lack in vivo activity of this nature, but only that the question remains undecided at present.

Because of the failure of any solution used in Experiment II, Part 1, to show protective action against histamine shock, we are unable to draw any definite conclusion regarding protective effect by saline injections. However, it is interesting to note that the dose of 8 ml. per kilogram is of the same relative order of magnitude as that used in intravenous saline therapy for surgical and other shock in man.

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# MELANOTIC RETICULAR HYPERPLASIA OF LYMPH NODES\*

## REPORT OF A CASE

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**B**IOPSY, whenever possible, has become a routine procedure to establish a diagnosis which cannot be made with certainty by any other means. This is especially true in the difficult group of diseases characterized by lymph node enlargement. It is important to bear in mind that local lymph node enlargement may not necessarily have any relation to a systemic illness of which the patient complains. The purpose of this paper is to report a characteristic lymph node pattern which was found accidentally in a young adult who died of acute pulmonary military tuberculosis. This benign lesion has some superficial histologic resemblances to the malignant lymphoblastomatous and lymphogranulomatous lesions with which, indeed, it was confused until the true nature of the lesion was described by Pautrier and Woringer.<sup>1</sup> It is, therefore, worthwhile to call attention to this generally unfamiliar, although probably not uncommon, lesion.

## REPORT OF CASE

K. T., a young negro boy, 17 years old, entered the Temple University Hospital on the service of Dr. Charles L. Brown on Sept. 14, 1940. He was well until June 1, 1940, when he noticed sharp stabbing pains around the heart, which lasted ten to fifteen minutes, and which were at times so severe as to cause him to faint. He was put to bed by his doctor for three weeks. At the expiration of this time he found that he was too weak to stand and was very short of breath. Three weeks before admission he developed pains in his extremities, especially the lower ones, and in his joints. Since the onset of his illness he lost 50 pounds. All during his illness he believes that he had fever, which occasionally was as high at 104° F.

Physical examination revealed a young adult negro male who had evidently lost considerable weight. There was nothing of note in the entire examination, except for the chest and inguinal lymph nodes. Expansion was limited and lagged on the left side of the chest. There was impaired percussion on the left side posteriorly from T<sub>8</sub> downward; this extended anteriorly to the midaxillary line. Breath sounds were absent in this region. There was moderate enlargement of the inguinal lymph nodes on both sides. These lymph nodes were discrete, not tender, and varied in size up to 5 cm. in diameter.

The laboratory findings were as follows: hemoglobin varied from 11.5 Gm. to 13 Gm. (Haden-Hausser significant). R.B.C. 4.01 to 4.83 million, W.B.C. 6,300 to 5,700. The average differential was neutrophils 74 (N. F. 47), lymphocytes 17, monocytes 6, eosinophile 1, neutrophilic myelocytes 3. Urine was slightly positive for occult blood, 6 to 7 R. B. C. per high-power field, W.B.C. 3 to 4 per high-power field occasional coarse granular casts. The electrocardiogram revealed a simple tachycardia with low T-waves, suggestive of some impairment to myocardial function, probably secondary to the patient's general infection. During his stay in the hospital he ran a continuous fever, ranging from 99° to 105° F. X-ray examination of the chest revealed an abnormal diffuse punctate type of density throughout both lung fields, and a small fluid collection in the left pleural space. Intravenous pyelogram presented a normal x-ray appearance.

\*From the Department of Medicine, Temple University Medical School, Philadelphia.  
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The presumptive diagnosis was tuberculosis. However, sputum, gastric washings, urine, and cerebral spinal fluid studies were repeatedly negative for tubercle bacilli. Because of the inability to establish a definite diagnosis, it was decided to remove one of the inguinal lymph nodes for histologic study.

The lymph node removed was pigmented and slate gray in color. It was completely encapsulated. In one section at one pole there was a single island of giant cells, suggestive of tuberculous origin. The remainder of the gland had none of the characteristics of tuberculosis. There was considerable thickening of the capsule. The follicles were small and poorly defined and had been replaced apparently by a tremendous hyperplasia of the reticulo-endothelial cells. These cells were polymorphous in appearance with occasional prominent and multiple nucleoli. The most striking feature, however, was the tremendous infiltration of fat and melanin within the reticulo-endothelial cells.

The patient died on Oct. 11, 1910, and autopsy disclosed an acute pulmonary miliary tuberculosis with terminal miliary spread to the other viscera.



Fig. 1.—Illustrating thickened capsule, disorganization of normal architecture, marked reticulum cell hyperplasia with deposition of fat and melanin.

#### DISCUSSION

The major portion of the lymph node picture was not obviously due to tuberculosis. The tremendous amount of fat, melanin, and reticular hyperplasia represented a puzzling picture until the contribution of Pantrier and Woringer was discovered. They found a rather characteristic histologic picture of lymph nodes in a variety of dermatologic diseases, the common denominator of which was pruritus. They called this lesion "La reticulose lipomelanique." The description of this lesion is taken rather freely from their contribution.

The lymph nodes are normally encapsulated and free of adhesions. They are moderately enlarged up to the size of a pigeon's egg. Upon sectioning, the cortical zones appear clearer than normal, with scattered brown flecks representing melanin deposits. Histologically, the lymph node tends to retain its fundamental architecture. The lymph follicles are smaller than normal and

are separated farther apart than normal. This is due to the interfollicular reticulum cell hyperplasia, which causes a gradual reduction and ultimate disappearance of the secondary lymph follicles. The germinal follicle, however, remains active and may be larger or smaller than normal, depending upon the extent of reticular hyperplasia. The reticular cells are predominantly cortical in position, although with increase in production, they may invade the medulla. The reticular cells are large pale cells with indefinite cytoplasmic borders. The cytoplasm appears faintly vacuolated with an empty meshwork. The nuclei are large, clear, elongated, or irregularly oval, with sprayed chromatin and occasionally two prominent nucleoli. These cells contain large quantities of lipid substances easily stained with sudan III or schiarlach R and are, according to

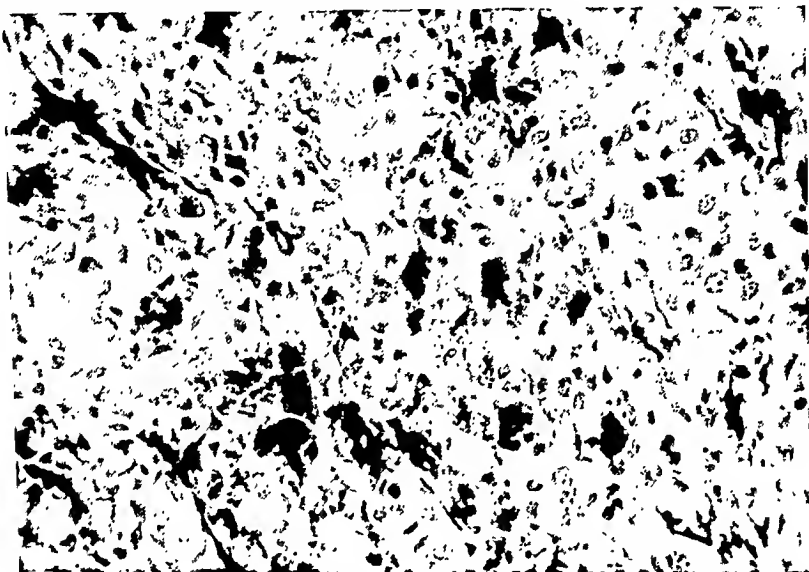


Fig. 2.—High power. Illustrating the polymorphous reticulum cell hyperplasia. The black deposits represent melanin. The clear spaces represent fat.

Pautrier and Woringer, cholesterol and double refractile. In addition, in the peripheral reticular cells especially are deposited large amounts of brown pigment, which by staining can be identified as melanin. The entire vascularity of the gland is increased. The marginal subcapsular sinuses are dilated and free for lymphocytic circulation. Eosinophiles may be found in them.

Pautrier and Woringer interpreted the hyperplastic proliferation of the reticular cells as due to an attempt of these cells to engulf and dispose of the excessive lipid substances and melanin. They found these lesions in persons subject to a variety of skin diseases associated with pruritus. Normally, melanin, which is elaborated by the melanoblasts in the basal layer of the skin is expelled toward the surface by the normal process of keratinization. A minute histologically undetectable quantity may travel toward the regional lymphatics and ultimately be excreted by the kidneys, and perhaps by the intestines. With scratching induced by itching, more than a normal quantity of melanin is formed. The regional lymphatics are dilated by the irritation. These factors, in addition to the massaging action of scratching, help to drive excessive quan-

tities into the regional lymphatics, where it is trapped by the reticular cells and causes them to multiply. They believe that the lipoid process is similar. The amount of melanin deposition varies with the duration and the severity of the skin lesion. It probably also varies with the melanin content of the skin.

In the absence of considerable melanin deposition, the lymph node may bear a striking resemblance to Hodgkin's disease. Dorothy Reed cells are not present, but the reticular cells are highly polymorphous and voluminous at times. The nucleoli are prominent and may be multiple; eosinophiles, plasma cells, and neutrophils may be plentiful in the subcapsular lymphatics; fibrosis may be present in the older lesions, and a partial or complete disorganization of the lymph node architecture appears. Indeed, the diagnosis of Hodgkin's disease, mycosis fungoides, and reticulum cell sarcoma had been made on many tissues which Pautrier and Woringer later diagnosed as "La réténose lipomelanique."

After histologic examination of the lymph node, the patient was asked specifically if he ever had any skin disorders. He stated that for several years he has had itching, occasionally scaling lesions in both groins for which numerous remedies had been tried unsuccessfully. These lesions were thought to be due to his occupation, which was that of pressing suits with a steam pressing machine. This required him to place one foot alternately on a foot stool. Apparently the heat and friction were sufficient to set up this irritation. He frequently attempted to obtain relief from the itching by scratching. He had been doing this work irregularly for the preceding four years and about two years before, he noticed the appearance of "lumps" in his groins.

#### CONCLUSION

A specific lymph node picture distinguished histologically by a marked reticulum cell hyperplasia rich in fat and melanin occurring in a variety of diseases characterized by itching is reported.

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# LABORATORY METHODS

## GENERAL

### THE OPSONOCYTOPHAGIC TEST IN ACUTE DIARRHEA IN INFANTS AND CHILDREN\*

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FOR several years extensive bacteriologic studies have been made of the stools of infants and young children who were admitted to the Children's Hospital of Cincinnati suffering from acute diarrhea. Stools from 50 per cent of these patients have contained *Shigella paradysenteriae*<sup>1</sup> (dysentery bacilli), but repeated stool examinations have offered no information regarding the etiologic factor in the patients from whose stools *Shigella paradysenteriae* could not be isolated. It was hoped that a determination of the opsonocytophagic power of the blood of such patients for the organisms isolated from their stools might indicate a possible causal relationship between the presence of these organisms and the disease. This theory was not substantiated by observation in our group of patients—high indices being obtained from patients both with and without diarrhea. The results from the patients with dysentery, however, were of considerable interest and, accordingly, are being reported here.

#### METHODS

The method employed for studying the opsonocytophagic index was for the most part an adaptation of the method of Huddleson, Johnson, and Hamann,<sup>2</sup> and consisted of mixing equal quantities of citrated whole blood and standardized formalin-killed bacterial suspension in agglutination tubes, incubating for thirty minutes at 37° C., mixing, streaking as for a differential blood count, staining with dilute crystal violet, and determining the number of organisms in fifty polymorphonuclear leucocytes. Whole blood was used throughout these studies, because previous investigations by others<sup>3, 3</sup> indicated that there is thus obtained a more accurate indication of the actual phagocytic response to bacterial invasion than when serum plus washed leucocytes of another individual or species is employed.

Sodium citrate was used as an anticoagulant, since, in our experience, heparin was not so satisfactory. Two and a half cubic centimeters of blood were added to small tubes containing 0.1 c.c. of a 20 per cent citrate solution in 0.9 per cent sodium chloride. The final concentration of citrate was 0.77 per cent. The specimens were kept in the refrigerator to prevent disintegration and were

\*From the Children's Hospital Research Foundation, Cincinnati, Ohio, and the Department of Pediatrics, University of Cincinnati.  
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used within four hours after they had been obtained from the patient. Specimens were thoroughly mixed just before using.

The formalized bacterial suspensions<sup>1</sup> were prepared by washing the growth from several twenty-four-hour-old brain agar slants with dilute formalin (0.4 c.c. of commercial formalin to 100 c.c. of a 0.9 per cent sodium chloride solution). After these suspensions of bacteria in formalin had been allowed to stand at room temperature for twenty-four hours, they were washed by centrifuging, decanting, and resuspending in fresh 0.9 per cent sodium chloride solution. This process was repeated three times. The density of the suspensions was standardized by the method of Gates,<sup>2</sup> so that a wire loop could be seen 2 cm. below the surface. Approximately 0.1 per cent formalin was added to the standardized suspensions as a preservative.

The phagocytic tests were set up in small, sterile agglutination tubes. The citrated whole blood (0.1 c.c.) and the formalized bacterial suspension (0.1 c.c.) were mixed by shaking the racks of tubes twenty times, after which they were placed in an incubator at 37° C. At the end of thirty minutes the tubes were removed, the contents were mixed by tapping gently twenty times, and the tubes were placed in an ice-water bath in order to check further phagocytosis. A small amount of the mixture was removed from each tube with a capillary pipette and smears were prepared as for a differential blood count. The best preparations were those on which the smear thinned out and terminated near, or just beyond, the center of the slides. It was found advisable to dry the smears rapidly by fanning at room temperature in order to avoid distortion of blood cells.

Most of the stains recommended by other workers did not appear to be satisfactory; some of them failed to stain the bacteria well, some stained the red cells too deeply, and others gave a hazy, indistinct appearance to the entire slide. Eventually, it was found that crystal violet gave excellent stains when used in the following manner: Smears were fixed momentarily in absolute methyl alcohol. While still dripping with alcohol, they were immersed in freshly prepared dilute crystal violet (1 drop of saturated alcoholic solution per cubic centimeter of distilled water) for approximately ten seconds. They were rinsed immediately in running tap water and dried rapidly in air. The bacteria stained deep purple; the white cells, lavender; and the red blood cells, bluish green.

Parenthetically, it may be noted that this method of determining opsonocytophagic power was satisfactory when used in the study of patients suffering from typhoid fever. The stained smears were examined by means of the oil-immersion lens. Most of the leucocytes were found along the sides and terminating edges of the smears. The number of phagocytized organisms in fifty polymorphonuclear leucocytes was noted and the average was then determined.

#### STUDY OF PATIENTS WITH ACUTE GASTRO-ENTERITIS

A number of different organisms were isolated from the stools of infants and children suffering from acute gastro-enteritis, in whose stools no members of the *Shigella* group were found. *Escherichia coli* were isolated from the stools of each of twelve patients; *Streptococcus faecalis* and organisms in the *Aerobacter*

group from approximately two-thirds of the patients; members of the *Salmonella* group from one-third of the patients; and members of the *Proteus* group from two patients. The phagocytic counts varied greatly for these organisms, and the high indices were distributed at random throughout the gastro-enteritis group and the control group without diarrhea. In none of these patients could any significance be attached to a high phagocytic index.

TABLE I

OPSONOCYTOPHAGIC INDEX ON BLOOD OF PATIENTS WITH DYSENTERY COMPARED WITH PATIENTS WITHOUT DYSENTERY USING THE SAME ORGANISM

| DYSENTERY PATIENTS |  | NONDIARRHEA PATIENTS |                        |
|--------------------|--|----------------------|------------------------|
| PATIENT            | OPSONOCYTOPHAGIC INDEX<br>HOMOLOGOUS CULTURE | PATIENT              | OPSONOCYTOPHAGIC INDEX |
| D. H.              | 9.6  | B. O.                | 0.3                    |
| J. H.              | 21.5   | B. O.                | 0.2                    |
| R. S.              | 37.5   |                      |                        |
| W. W.              | 4.2  | R. O.                | 0.4                    |
| R. W.              | 9.6*   | D. A.                | 0.1                    |
|                    |  | J. C.                | 0.3                    |
|                    |  | R. E.                | 0.4                    |
|                    |  | R. E.                | 1.1                    |
| P. B.              | 9.3  |                      |                        |
| S. D.*             | 1.0*   |                      |                        |
| R. D.              | 1.4  | H. M.                | 0.6                    |
| F. M.              | 27.2   |                      |                        |
| H. M.              | 22.8   | D. P.                | 0.1                    |
|                    |  | F. B.                | 0.1                    |

\*Four weeks after onset of disease.

#### PATIENTS WITH DYSENTERY

If the phagocytic counts on the blood from ten patients whose stool cultures contained *Shigella paradysenteriae* are compared with those from a group of patients without dysentery, as in Table I, it is seen that the blood specimens from all the patients with dysentery tested gave increased counts with the *Shigella* antigens, whereas none of the blood specimens from the patients without diarrhea showed any increase. Counts above 0.5 were considered to be significantly increased. The blood was secured during the acute stage of the disease, except when otherwise noted in the tables. Variations in the opsonocytaphagic index on blood of the patients with dysentery are seen in Table II, showing the tendency to increase during the acute stage and to decrease with convalescence.

TABLE II

PHAGOCYTIC STUDIES ON BLOOD OF FIVE DYSENTERY PATIENTS DURING ACUTE STAGE AND AT LATER PERIODS

| PATIENT | INDEX<br>ACUTE STAGE | TIME OF<br>SECOND TEST | INDEX |
|---------|----------------------|------------------------|-------|
| J. H.   | 21.5                 | 4 mo.                  | 7.5   |
| W. W.   | 4.2                  | 19 days                | 0.2   |
| R. W.   | 0.7                  | 1 mo.                  | 9.6   |
| R. D.   | 1.3                  | 2 mo.                  | 0.8   |
| H. M.   | 22.8                 | 8 days                 | 26.2  |
| E. M.   | 16.3                 | 5 days                 | 28.1  |

#### STUDY OF A FAMILY WITH DYSENTERY

Interesting observations were made of the phagocytic counts obtained with blood from several children in one family, several members of whom had

dysentery (Table III). *Shigella paradysenteriae* of the Flexner type were isolated from the stools of four of these children, Elizabeth, Eugene, Florence, and Harriet, only the last of whom was severely ill. Two other siblings, George and Alice, also had diarrhea, but no *Shigella paradysenteriae* were isolated from their stools. The other sibling, Jennie, had no diarrhea, and no organisms of the *Shigella* group were found in her stools.

TABLE III  
PHAGOCYtic STUDIES IN A FAMILY IN WHOM DYSENTERY DEVELOPED

| PATIENT   | DIARRHEA | STOOL CULTURE | SHIG. PARADYS-ENTERIAE (HARRIET) | E. COLI (FLORENCE) | E. COLI (HARRIET) | AEROBAC-TER AEROGENES (HARRIET) | STREP. FAECALIS (FLORENCE) |
|-----------|----------|---------------|----------------------------------|--------------------|-------------------|---------------------------------|----------------------------|
| Elizabeth | Yes      | Positive      | 28.1                             | 42.5               | 0.7               | 0.5                             | 2.6                        |
| Eugene    | Yes      | Positive      | 16.3                             | 16.3               | 4.6               | 0.3                             | 1.5                        |
| Florence  | Yes      | Positive      | 8.2                              | 37.6 (0.8*)        | 0.7               | -                               | 2.5                        |
| Harriet   | Yes      | Positive      | 26.2                             | 4.5 (0.5*)         | 1.9 (0.6*)        | 0.5                             | 1.4                        |
| Alice     | Yes      | Negative      | 28.2                             | 29.0               | 7.6               | 0.6                             | 1.9                        |
| George    | Yes      | Negative      | 23.4                             | 12.5               | 1.9               | 0.6                             | 1.4                        |
| Jennie    | No       | Negative      | 0.4                              | 0.6                | 1.3               | -                               | -                          |
| N. P.     | No       | -             | 0.1                              | 0.8                | 0.3               | 0.2                             | 0.7                        |
| N         | No       | -             | 0.8                              | 4.7                | -                 | -                               | -                          |
| M. S.†    | No       | -             | 2.2                              | 23.2               | 0.7               | 0.5                             | 1.9                        |

\*Six months later.

†Infant with scalp infection.

The four siblings who had dysentery, and the two who had diarrhea with negative stool cultures, gave high phagocytic counts with the *Shigella paradysenteriae* recovered from Harriet's stool. Jennie, who did not have a diarrhea and whose stool culture was negative, showed no increase in her phagocytic count. In the lower part of Table II it is seen that the blood of the patients without diarrhea did not have an elevated index for the *Shigella paradysenteriae*, except in the instance of M. S., an infant with furunculosis of the scalp, whose index was only slightly above normal.

#### SUMMARY

The whole blood from patients suffering from acute infections with members of the *Shigella paradysenteriae* group gave increased opsonocytophagic counts with antigens of these organisms. Blood from patients without diarrhea did not give increased counts with such antigens.

Blood from patients suffering from infection with *Shigella paradysenteriae* and from those with diarrhea whose stools did not contain these organisms gave high phagocytic counts at times with various bacteria other than *Shigella* isolated from their stools.

These tests do not furnish evidence suggesting that any particular bacteria might be the causative agent in those children suffering from diarrhea whose stools are negative for the *Shigella* group.

The opsonocytophagic test aids in the diagnosis of infection with *Shigella paradysenteriae*, but it is not a necessary test when it has been demonstrated that the stools of patients contain these organisms. A negative opsonocytophagic test (counts less than 0.5) probably indicates the absence of dysentery infection.

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## A NEW METHOD OF STAINING WITH WRIGHT'S SOLUTION\*

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THE primary requisite of a good method of staining is its capacity to reveal clearly the various structures in the blood film in order that they may be easily distinguished from one another. Simplicity, practicability, and rapidity may also be added as indispensable qualities for clinical purposes.

The method now suggested carries these features. It is as follows:

1. Cover the dry smear with a few drops of Wright's staining solution.
2. *With the breath* blow the smear as uniformly as possible until it becomes pinkish violet in color. This process will consume about ten seconds. The harder one blows, the faster will be the fixation of the stain.
3. Wash with tap water.
4. With the water running slowly over the slightly tilted slide, pour a few drops of Wright's stain. The contact between it and the smear should be made as short as possible. A long contact may decolorize the smear.
5. Wash, dry, and examine under oil immersion.

Two new features have been introduced: (1) the blowing of the smear with the breath, and (2) the washing of the stained smear with the staining solution itself. The first accelerates the process of staining, reducing its entire duration from minutes to seconds. The second dissolves the precipitated particles left in the course of staining. It thus solves one of the important objections to Wright's stain, i.e., precipitation of the solution on the slide.

Another decided advantage of this method is that in case the hematologist is not satisfied with the shade of color obtained at the first attempt, he may rectify it without much loss of time. If the smear is understained, he may repeat step 2; if overstained, step 4. It must be remembered that Wright's staining solution is a decolorizing as well as a coloring agent, depending upon the duration of its contact with the smear. This method renders the blood film

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flexible in the hands of the examiner. The process of restraining and decolorizing may be repeated several times on the same smear without the slightest injury to the cells.

If a more thorough study of the red blood cells and the platelets is desired, the following procedure may be inserted between steps 3 and 4:

3a. Dry the film, put one drop of cedarwood oil over it. Add xylol to dissolve and with the palmar surface of the small finger cover the whole slide with the solution, in the same way as when cleaning a slide after use. Dry and proceed to step 4.

This modification, which turns the film pink in color, is useful also in the examination for malarial parasites and in counterstaining after brilliant cresyl blue. It deepens the contrast between the malarial bodies, or the "net" structures and the pinkish cytoplasmic background.

I have employed this method with good results in staining smears secured from the peripheral blood and the bone marrow. I have found that it has all the advantages of Wright's and Giemsa's stains minus their objectionable features.

#### SUMMARY

A new method of staining with Wright's solution is proposed. It is simple, very rapid, practical, and convenient. It does not leave precipitates on the slides and shows structures very distinctly. A modification of the same for the study of the erythrocytes, platelets, reticulocytes, and malarial parasites is also recommended.

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### A METHOD FOR STOMACH TUBING SMALL ANIMALS\*

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RECENTLY we were confronted with the problem of establishing a technique suitable for the prolonged oral treatment of a large number of rats. The procedures used by others seemed both unphysiologic and too time-consuming, and in addition entailed unnecessary risks. For example, a method necessitating the use of an anesthetic requires too much time and may have unphysiologic sequela. In our trials the use of a semirigid catheter as a stomach tube proved unsatisfactory, since the material was readily nicked by the teeth of the rat and the sharp edges of these cuts were obviously a source of irritation to the esophagus. The splendid results obtained by the method of Pugh and Tandy,<sup>1</sup> which employs a modified hypodermic needle, are indicative of a highly feasible method. Nevertheless, we wished to simulate more nearly the proved procedure employed for human work.

After trying these procedures with modifications, we finally developed a method which involves not only a minimal number of manipulations on the part of the operators, but also relatively little discomfort to the rat.

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During a period of six months some 200 animals of various ages have been subjected to this procedure for varying lengths of time, representing approximately 4,000 treatments without the loss of a single animal from trauma or drowning.

The procedure we have developed is easily and rapidly performed, and entails no deleterious effect to the animal.

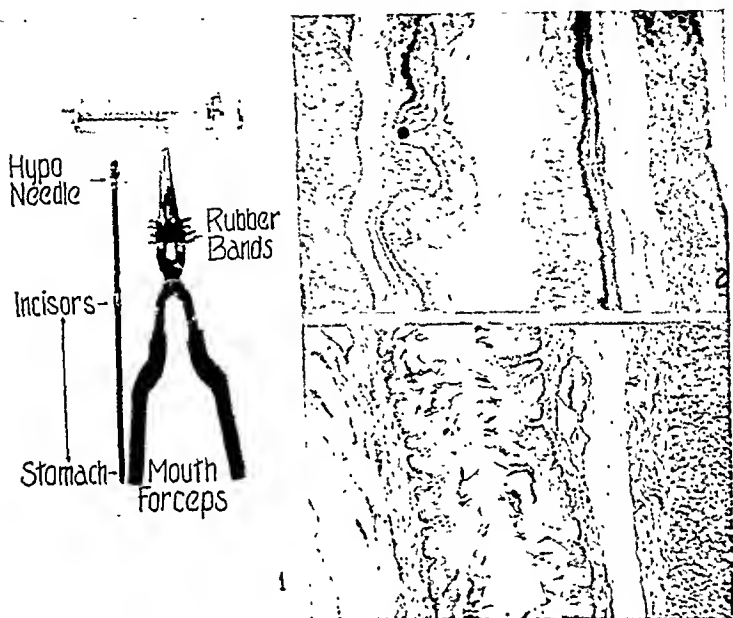


Fig. 1.—Shows the instruments used for this method.

Fig. 2.—The esophagus of an animal treated by this method three times weekly for twelve weeks (enlarged 50 times).

Fig. 3.—The esophagus of an untreated animal (enlarged 50 times).

#### APPARATUS

In order to open the rat's mouth and at the same time have complete control of its jaws during the procedure, the forceps shown in Fig. 1 was developed. The instrument, called a needle nose pliers, may be procured from any automobile supply store for a nominal sum. The original spring attached between the handles was considered too stiff and was, therefore, removed. To develop the necessary tension rubber bands were substituted and wound around the blades just distal to the fulcrum. In this manner the amount of tension may be nicely adjusted to please the operator.

A No. 8 or 10 soft rubber catheter was employed as a stomach tube. A piece of adhesive tape wrapped several times around the catheter not only marks the distance it is to be passed, but also protects the tube at that point from the animal's teeth.

A large No. 12 gauge hypodermic needle, which snugly fits the catheter, was used to attach it to the syringe.

## PROCEDURE

The procedure requires two operators, one to hold the animal and open its jaws with the forceps, and the other to pass the stomach tube and administer the medication.

The animal is held gently but firmly, its head projecting upward between the thumb and forefinger. Excessive motion is prevented by extending the remaining fingers along the belly of the rat. With the other hand the operator inserts the blades of the forceps between the jaws just behind the incisors and applies pressure sufficient to maintain the mouth in a suitable position for passage of the stomach tube. The other operator then proceeds to pass the tube by running its tip along the roof of the mouth to gain entrance into the esophagus, and with gentle pressure pushes it downward until the strip of adhesive tape is at the level of the incisor teeth.

One of the advantages of this method is that the operator is able to feel any abnormal resistance that the tube may encounter in its course and is thus able to prevent injury or death of the animal. If this condition occurs when about two-thirds the measured distance of the tube has been inserted, one may feel reasonably certain that the trachea has been entered. If, however, the hindrance is beyond this point, it is almost certain to be at the cardiac sphincter. In either case it is best to remove the tube entirely and to repeat the process after the animal has rested several minutes.

In order to ascertain whether, after a prolonged period of treatment by this method, any unwarrantable conditions developed, each esophagus and stomach was subjected to a careful examination. Some one hundred examinations showed no gross pathology. Subsequent histologic studies revealed only one case of a traumatic ulcer. The esophagus shown in Fig. 2 is typical of an animal treated three times weekly for twelve weeks. Fig. 3 shows the esophagus of an untreated animal.

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## THE MICROSEDIMENTATION RATE\*

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THE usefulness of the sedimentation rate is well established in following the course of rheumatic fever and rheumatic heart disease. It has been widely studied in tuberculosis and in many other conditions. Interest in the test appears to be increasing, and the desirability of a clinically suitable micromethod would seem evident.

It is the very multiplicity of methods for the sedimentation rate that has probably kept this test from having the widespread clinical use that it deserves. Each method has required its own normal standards, and in the case of micromethods, these "normals" have often been inadequately derived, and unverified by others who have accepted them. This may explain why some authors consider that an adequate micromethod does not exist.

Recently, a monograph on sedimentation considered only venipuncture methods, and showed that the small diameter tubes of any micromethod would have a greater inaccuracy than the larger macrotubes.<sup>4</sup> This need not prevent the use of the micromethod clinically, although it might well interfere with the study of the nature of the sedimentation phenomenon. Uneven settling of the column of red blood cells, probably the chief criticism against the use of the narrow microtubes, has rarely occurred in my studies. (Irregular settling, whether in large or small tubes, is sometimes due to reticuloeytosis, as it has been shown that the reticulated red blood cells remain at the top of such unevenly settling columns.<sup>13</sup>)

Any method for sedimentation must provide for collection of blood with the use of an anticoagulant, adequate mixing, and suspension of the blood column in a strictly vertical position. As long as this required two to four or more pieces of glassware, and as many maneuvers, no method, micro or macro, was likely to get into widespread use in the office or clinic. Seven years ago Landau<sup>8</sup> published his micromethod, which was simple to the point of encouraging frequent use. I have worked with it since 1934, and wish to report certain further simplifications (but not fundamental alterations) of the method, as well as the results of my clinical studies and the normal values established with its use.

Since this is not fundamentally a new method, the results are directly comparable with previous studies.<sup>3, 5</sup> The results are not comparable with other methods unless the height of the blood column in the tube and the method of

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TABLE I  
COMPARISON OF PREVIOUSLY DESCRIBED MICROMETHODS

| AUTHOR                            | YEAR | NORMALS   | LENGTH OF BLOOD COLUMN  | BORE OF TUBE    | ANTI-COAGULANT  |
|-----------------------------------|------|---|---|-----------------|---|
| Landau <sup>8</sup>               | 1933 | 1 to 8 mm. in 1 hour (174 tests on 78 children; 1 to 5 mm. in 70 men; 1 to 8 mm. in 80 women)   | 62.5 mm.  | 1.0 mm.         | 5% sodium citrate   |
| Elghanmer <sup>3</sup>            | 1934 | 3 to 8 mm. in children  |   | Landau's method |   |
| Cutler <sup>2</sup>               | 1927 | 2 to 8 mm. for men; 2 to 10 mm. for women. (Comparable to his macromethod, which also uses a 50 mm. blood column height; 5,000 patients were studied by the macromethod)  | 50.0 mm.  | 2.5 mm.         | 5% sodium citrate   |
| McSweeney <sup>9</sup>            | 1934 | 10 mm. or less for normal children and rheumatic children in a quiescent period   | 100.0 mm.   | 1.0 mm.         | 3.5% sodium citrate   |
| Beaumont and Maycock <sup>1</sup> | 1935 | 0.5 to 4 PER CENT in one-half hour for men, and 1 to 8 PER CENT for women   | 100.0 mm.   | 1.13 mm.        | 3.5% sodium citrate   |
| Smith <sup>12</sup>               | 1936 | "Within the range usually stated by the Cutler venipuncture method for normal adults." Did 45 tests on 42 patients, but only 18 tests on patients 8 years old or over   | 50.0 mm.  | 2.5 mm.         | 5% sodium citrate   |
| Rogatz <sup>11</sup>              | 1938 | "All observers have found that a normal sedimentation value lies between 5 mm. in one-half hour and 10 mm. at the end of 1 hour." Reported only 7 tests on children 8 years old or over, and only 50 tests in all |   | Smith's method  |   |
| Vollmer <sup>14</sup>             | 1939 | 8 to 10 mm. in 1 hour. Over 15 mm., "marked acceleration." Did 104 tests on 52 children   | 44.5 mm.  | 2.5 mm.         | 3% sodium citrate   |
| Kato <sup>6, 7</sup>              | 1940 | 0 to 20 PER CENT  | 64 to 102 mm. (Whatever the tube dimensions, a volume of 50 c.mm. of blood is constant) | 0.79 to 1 mm.   | Special potassium oxalate formula "approaches heparin in results" |

recording are the same (see Table I). The attempt to compare different methods by recording the millimeters of settling as percentages of the original blood column height is only approximately accurate, since changing the height of the original blood column does not necessarily change in like degree the observed sedimentation distance.

Landau reported his readings as *millimeters in one hour* (i.e., millimeters of fall of the top of the settling erythrocyte column). In my experience, this method is clinically adequate and preferable to other methods of reporting, such as millimeters per minute during the period of greatest fall, arbitrarily chosen index or percentage systems, or the time required to fall some given distance. A graph can be made of the rate of fall by five-, ten-, or fifteen-minute periods during the hour, but this is rarely useful, and certainly not conducive to doing many tests at one time, as in a clinic. Occasionally comparison of the thirty-minute value with the final reading appears to be useful.

The normal values previously established have been open to question. Landau's original material consisted of 78 children, admitted to the hospital for various causes, and found, after careful examination, to be without demonstrable symptoms of disease.<sup>8</sup> On these patients 178 tests were done. Landau adds that "we had ample experience among polyclinic patients. The normal values for children over two years of age may vary from 1 to 8 mm. without any pathological condition being present. We have been unable to discover any noteworthy difference in value for different sexes with any degree of certainty. Our impression is, however, that as a general rule the normal values do not exceed 5 or 6 mm. But it is of great importance in such a phenomenon as sedimentation not to draw too narrow limits for the normal values." In 80 healthy women of the hospital staff he found values of 1 to 8 mm., and in 70 men, values of 1 to 5 mm.

The next year Elghammer,<sup>3</sup> using Landau's method, stated that "values of 3 to 8 mm. are to be regarded as normal rates in children; 8 to 11 constitutes the zone of possible pathologic indication, 11 to 25 a definitely increased rate, and 25 to 50 a marked acceleration."

No subsequent studies by this method have appeared in the American or English literature. Seven other micromethods have been described, each with the fault either of needless complexity of technique, or of inadequately derived normal values, or both. These are summarized in Table I, along with Landau's and Elghammer's results.

In the present study with Landau's method, about 2,600 tests were available for analysis. Experience soon showed that acceptance of previous normal standards was causing confusion and apparent unreliability. An attempt was made, therefore, to derive normal standards, statistically, and in patients clinically verified to be normal as far as activity of rheumatic infection was concerned.

It is not my purpose to discuss the nature of the phenomenon of sedimentation, influence of various anticoagulants, the detailed comparison of micro-methods and macromethods, nor the question of the various so-called corrections. This has been well covered by others.<sup>4, 5, 12</sup> Landau's method has been found useful clinically without considering any corrections other than that of room temperature. This should be between 60° and 80° F.; if the room is 5° to 10° F. above this, the reading may be 2 to 4 mm. higher; if the room is correspondingly below 60° F., the reading may be 2 to 4 mm. lower than the "true" reading (see also Appendix at end of paper). Fortunately, the present trend of

investigators is to realize that "corrections" may well favor incorrect clinical judgments. Hambleton and Christianson,<sup>5</sup> and Kato,<sup>6</sup> have stressed this in particular.

Of the 2,600 tests mentioned, which were the basis of this study, 2,094 tests were made on patients, from 8 to 23 years old, with cardiac involvement. The immediate purpose was to establish "normal values" for this group, so as to make of the test a useful indicator for judging the presence or absence of active rheumatic infection. The following were the criteria for selecting tests for statistical analysis of the normal range:

1. Absence of any clinical evidence of active rheumatic infection or carditis at the time of the test.
2. Absence of any type of infection or recognized disease, other than the inactive rheumatic fever, at the time of the test.
3. Absence of any suspicion of such rheumatic activity or other disease or illness within a month before and after the given test.

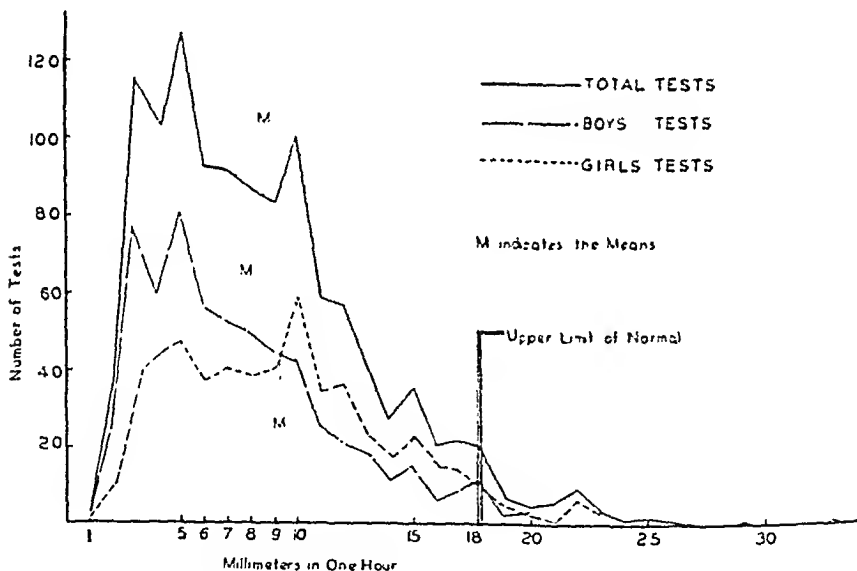


Fig. 1.—Distribution curve for determining normal values.

Tests not meeting these criteria were not included as a "normal" for statistical study even if they fell unquestionably within the normal range.

On the above basis, 1,165 tests were suitable for analysis, 620 on boys and 545 on girls. Of these, 352 were done personally; the remainder were done at the Sunset Camp for Cardiac Children by eight resident physicians under my supervision.\* In many instances, the children were seen year after year, at the camp and in the various clinics in Chicago.

Fig. 1 shows the distribution curve of these 1,165 tests. It is seen to be skewed to the left, but it is reasonable to accept it as being within the limits of a normal distribution curve. In such a case the data from which the curve

\*A special statistical study was made to demonstrate the fact that these residents learned the method quickly, and that their results were uniformly comparable and reliable.

is constructed may be used for the statistical derivation of a range of normal values. The basis for these statements is found in Table II, as well as the results of the statistical study. In a series of studies, one of the purposes of which was to establish normal values for a modified macrosedimentation method, Osgood and his associates<sup>10</sup> have shown that their observed values for the sedimentation rate also follow a skew curve.

TABLE II

RESULTS OF A STATISTICAL ANALYSIS OF 1,165 MICROSEDIMENTATION TESTS FOR THE DETERMINATION OF NORMAL VALUES

|  | TOTAL   | BOYS    | GIRLS   |
|--|---------|---------|---------|
| Number of tests                                  | 1165    | 620     | 545     |
| Range  | 1 to 33 | 1 to 33 | 1 to 27 |
| Mean   | 8.52    | 7.80    | 9.33    |
| Mode   | 5       | 5       | 10      |
| Standard deviation                               | 4.62    | 4.67    | 4.57    |
| Average deviation                                | 3.54    | 3.47    | 3.62    |
| Standard deviation divided by average deviation* | 1.31    | 1.34    | 1.26    |
| Error of mean                                    | ±0.14   | ±0.19   | ±0.20   |
| Upper limit of normal                            | 17.76   | 17.14   | 18.47   |
| Upper limit of normal for practical purposes     | 18      | 18      | 18      |

\*For a "normal curve," this quotient is usually considered to be 1.25 or less. The values here shown are trivially over the limit.

Inspection of Fig. 1 shows a range of one to 33 mm. in one hour. However, the upper limit of normal is not to be taken as 33 mm. The mean is 8.52 mm., and as seen in Table II, the standard deviation of the mean for all tests is 4.62 mm., with the standard error of the mean  $0.19\pm$ . In other words, the mean really is somewhere between 8.33 mm. and 8.71 mm., and the average variation from the mean of any given test is 4.62 mm.

For practical purposes the range of normal values is usually considered to be two standard deviations in each direction from the mean, when such data is available and forms a curve whose degree of skewness is within the limits of a normal distribution curve. Hence, 2 times 4.62 (the two standard deviations) plus 8.52 (the mean) equals 17.76 mm. Since the test is read only to the nearest millimeter, it may be stated that 18 mm. in one hour is the upper limit of normal for all tests.

The lower limit becomes less than zero, which would mean, statistically, that there was no such thing as too slow a rate. Actually, this is not the case, as will be discussed subsequently.

Theoretically, 95 per cent of all the tests are included in this total range of four standard deviations. Actually it here includes 97.5 per cent. Although the half dozen or so "unexplained" high values are almost certainly artifacts, they are not excluded from analysis.

Reference to Table II again will show that the upper normal limit for girls is slightly higher than for boys, and this is true also of the mean values. These differences can be shown to be statistically significant, but practically they are unimportant, being scarcely more than a millimeter. As stated previously, the test is read only to the nearest millimeter.

Twenty-five per cent of these tests are higher than 10 mm., the previously accepted normal limit. In reviewing the records of *all* those patients in the 11 to 18 mm. group, it is found that, first, many patients with mild upper respiratory infections and various minor conditions have normal rates, that is, 18 mm. or less. It has been an aid to clinical thinking to regard these as normal rather than as "slightly elevated" rates.

Secondly, and more important, no one with a rate consistently 18 mm. or less had *active* rheumatic fever or carditis at the time of the tests. Seeming contradictions to this are rare. The most important exception is congestive heart failure following acute rheumatic carditis, when the rate may fall to normal levels (perhaps because of liver damage with consequent decrease in blood fibrinogen).

No further comment is necessary about the well-recognized high rates in the presence of acute rheumatic fever or carditis, nor the fact that the high rate may have some other explanation.

The finding of a rate of 1 mm. or less should suggest first a clot in the tube. In its absence, the possibilities of polycythemia, liver damage, and finally, an allergic state should be considered. I have observed for some years an adolescent girl with mitral stenosis and true bronchial asthma whose sedimentation is usually 4 to 10 mm. When she developed a mild acute polyarthritis and carditis, her rate rose to 16 mm., regarded as abnormal for her.

There is a great value to repeated tests on each patient, so that the normal or control values may be established. In this way the significance of changes may be better appreciated. Normally, the sedimentation rate does not change more than a millimeter or two from day to day or week to week. Furthermore, there are no variations after eating, exercise, or bathing, such as cause confusion at times in leucocyte counts.

Serial rates are especially important in judging control values in middle-aged and elderly patients, in whom the diagnosis of "normal health" is always an increasingly difficult matter, and normal standards for sedimentation are harder to define. Approximately an additional 500 tests were studied in these older patients. For the present it has been found useful to apply the same normals as for the younger patients.

In the case of the rheumatic patients, the most difficult decisions as to management often come in that group with clinically borderline or questionable activity of infection. Herein the test has had its greatest practical usefulness. I have not been led astray by using this higher upper normal limit. It has served as an aid in deciding that in some cases 99.2° F. was to be regarded as the normal mouth temperature; in others that a heart rate of 100 did not depend on active carditis; in still others that loss of weight and appetite were not indications of recurrent rheumatic infection.

It should be restated that these comments are valid *at the time of the test*. In fact, the finding of a normal rate aids in interpreting the events of the past week or weeks rather than in foretelling the future. A normal rate may rise to 35 or more several days after the onset of an acute infection. In my experience

an equally rapid fall from high levels has not been encountered; several weeks or more are required after the acute episode is clinically over; indeed, sometimes it is several months.

Conversely, the finding of an abnormally high rate must be taken seriously, and an effort should always be made to find the reason for it. Increased sedimentation is not usually the earliest evidence of active rheumatic infection, but it may be, especially in the case of recurrences. Other significant signs or symptoms will appear in a week or two, rarely as long as a month. A preceding upper respiratory infection may have been responsible for this apparent "earliest evidence," but all upper respiratory infections are not followed by rheumatic activity; hence the rate may return gradually to normal. However, rheumatic reactivations are by no means all preceded by upper respiratory infections, so that the clinical reason for the early increase of sedimentation (before fever or tachycardia appears) may not be readily explainable.

#### SUMMARY

In a study of nearly 2,100 microsedimentation tests by Landau's method on 405 patients with cardiac disease, ranging in age from 8 to 23 years, values have been derived which are normal for inactive rheumatic infection, and probably for the age group as well. The upper limit of normal is 18 mm. in one hour, although the mean is 8.5 mm., and 75 per cent of the tests are 10 mm. or less. The results fall within the limits of a normal skew curve, permitting statistical analysis.

The Landau micromethod is advocated because of its simplicity and the absence of need for applying corrections.

No contradictions have been encountered to the rule that a rate of 18 mm. or less eliminates active rheumatic infection or carditis at the time of the test, with rare and fairly well understood exceptions, the most important of which is congestive heart failure following acute carditis. Conversely, a high rate must always be taken seriously, even in the absence of other signs or symptoms, and an effort must be made to find the reason for the high rate.

The possible significance of very low rates is discussed.

Approximately an additional 500 tests were studied in patients without rheumatism and in middle and elderly age groups; in the latter the diagnosis of "normal health" is always an increasingly difficult matter, and normal standards for sedimentation are harder to define. For the present, it is found useful to apply the same normals as for the younger patients, with emphasis on the establishing of control values by serial testing of each patient.

#### APPENDIX

*Technique for Sedimentation Test.*—Prepare the finger as for a blood count; for this acetone is preferred. Insert the short end of the pipette into the rubber tip of the aspirating syringe. After the patient's finger has been stuck, draw 5 per cent sodium citrate solution into the tube to the lower mark (12.5 mm. from the tip), wipe off the excess from the outside, and insert the tip into the drop of blood on the finger. (The first, second, or third drop may be used, with or without squeezing; if the coagulation time happens to be rapid, the fourth drop may cause error.)

Draw the blood into the syringe by slowly unscrewing the plunger of the syringe; the blood mixes with the citrate as it is drawn up, and when the advancing column of fluid, regardless of whether it is blood, citrate, or blood-citrate mixture, reaches the second mark (50 mm. above the first mark), no more blood is drawn into the tube. Remove the pipette from the finger, draw the fluid column up several millimeters more, and then wipe off the tip of the pipette. It is easy and desirable to develop the practice of holding the assembled outfit of pipette and syringe in one hand during this phase of the procedure, during which the tube should be held nearly vertically.

Now mix the blood by drawing it into the bulb of the pipette so as to leave about 5 mm. still in the straight part of the tube, and then re-expel it. This is continued until the blood has been drawn into the bulb and expelled at least eight times. After the fourth time the tube may be rotated 180° about its long axis for subsequent mixings. During this time it is well to hold the tip of the assembled outfit somewhat lower than the syringe while the blood is being drawn into the bulb and re-expelled. This tends to prevent the formation of air bubbles in the blood. However, the fluidity of the blood is sometimes such that the tube must be kept nearly vertical at all times; otherwise the blood will be broken up in the bulb by air bubbles.

It is important to avoid handling the bulb with the fingers; to do so warms the air in the bulb. When the tube is finally set up at room temperature, the cooling of the bulb may draw the blood column partly back into the bulb. If this occurs, remixing is necessary. Occasionally one can manipulate the outfit to expel an air bubble which may have formed by holding the tip uppermost. If the blood column is intact for all but a millimeter or two at one end after final mixing, significant error does not result. Otherwise, or if more than one air bubble has appeared, the test may be "salvaged" by expelling all the blood into a small depression such as that of a hanging-drop glass slide, and then drawing it back slowly so as to exclude the bubbles. I wish to emphasize that in practice this salvaging maneuver is rarely necessary.

After these mixings, when the blood is in the straight part or bore of the tube, put a finger of the left hand tightly over the tip of the pipette, and slowly remove the syringe with an even, unscrewing motion. Remove the left finger and press a small ball of plasticine firmly against the upper end from which the syringe has just been removed. Turn the tube upright quickly and place it in the wooden block. This maneuver requires no more than twenty seconds after the mixing is stopped, and if as much as sixty seconds elapses, an error may be introduced. The time for setting up a test, from start to finish, need not be over one and one-half to two minutes.

When the blood is let into the bore of the tube for the final time, the apparatus is turned into a vertical position, and the blood is slowly expelled from the bulb, so that a minimal amount adheres to the walls of the bulb. The bottom of the column should be about 15 mm. from the tip—the exact point is immaterial. An accurate millimeter rule is used to measure the distance of settling.

One of the outstanding advantages of this technique is that the test can be set up at some later time (up to six hours) and can be readily transported, e.g., from patient's home to the office. In this event, after mixing and putting on the plasticine seal, one may carry the tube horizontally or nearly so. (A fountain pen box makes a useful container in the bag.) When it is subsequently to be set up, the following is the procedure:

Hold the tube horizontally. Remove the plasticine by drawing the fingers toward it along the outside of the tube from the bulb to the upper end; in this way, it is removed without squeezing any of the plasticine into the lumen. If the plasticine is unduly soft, the tube may be plugged in spite of precautions. In such a case, a fine wire may be inserted into the plugged end and the plasticine broken up sufficiently to permit use of the screw-suction syringe. This is an infrequent "complication."

When the plasticine is off, hold the tube horizontal, put a finger of the left hand firmly against the tip of the tube, and hold it there while the syringe is put on with an even screwing motion. There is now unequal pressure on the two ends of the blood column, which must be equalized before mixing. With the left finger still applied, unscrew the plunger of the



syringe about three-fourths turn, and remove the left finger cautiously, as though handling a volumetric pipette. If the blood column moves toward the tip, a little further unscrewing of the plunger is required, perhaps another one-quarter turn. If the blood column advances into the bulb, the plunger must be screwed in slightly. A little practice is required to do this without admixture of air bubbles or loss of blood, but the knack is quickly acquired.

Then proceed to mix as before, with this very important difference, that more mixing is required when the tube has been transported as already described. I have determined experimentally that 20 mixings in the bulb are not enough, and that 24 are usually sufficient. To be sure, then, mix the blood 32 times, eight times in each of the four quadrants of rotation about the long axis of the tube. This may sound like too many times, but it is accomplished in one and one-half minutes or less. When thus mixed, the tube is set in the wooden block, as previously described. I wish to stress again that the tube must be strictly vertical, and at a room temperature of 60° to 80° F. (A room temperature of 90° F. adds 2 to 4 mm. to the reading; the error is not constant, and depends upon what the original reading would have been; temperatures below 60° F. retard the fall by a like amount.) Furthermore, if the room temperature changes suddenly, for example, from 75° to 65° F. in a few minutes, the entire blood column will move in the tube. Error is thereby introduced, as settling cells tend to become mixed with the already clear plasma above the line of demarcation.

**Materials.**—Tubes of the Landau type and the "Gomco" screw-suction aspirating syringe are obtainable from commercial supply houses.\*

A wooden block, approximately 2½ inches by 1 by ¾ inch, with holes machine-drilled in it so as to have them strictly vertical, will accommodate the tubes. Eight or ten holes can be drilled in such a block by any machine shop or carpenter. Most of the holes should be drilled with a No. 12 standard bit. To take care of chance odd sizes in the external diameter of the tubes, two holes can be drilled with a No. 11 bit, and two with a No. 13. A thin brass plate on the bottom will prevent uneven wear which may tend to throw the holes out of vertical alignment. (It has been shown that as little as 2 per cent deviation from the vertical will accelerate the settling in the tube, and thereby introduce error.<sup>15</sup>) A small pocket-sized spirit level may be used to check the levelness of the table or whatever support is used for the block.

A small package of plasticine or modeling clay, and 5 per cent sodium citrate solution, complete the outfit.

The somewhat more elaborate apparatus originally described by Landau was imported, and so far as is known is not now obtainable because of war conditions. I have used the original outfit, but I find it no more accurate, and indeed, less convenient. It was, furthermore, very expensive, which is not the case with the assembled apparatus herein described.

Acknowledgment is made to the Sunset Camp for Cardiac Children for studies made there.

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104 SOUTH MICHIGAN AVENUE

## SIMPLE, SHIELDED BIPOLAR ELECTRODES\*

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IN 1933 one of us (B. C.), working in this Laboratory, described a method for stimulating autonomic nerves in the unanesthetized animal by means of implanted electrodes.<sup>1</sup> Because of their flexibility these electrodes were able to withstand the strain of being imbedded in moving parts for a considerable period of time, and, because soft rubber was used throughout, there was no injury to the nerve or adjacent tissue. During the intervening years a bipolar modification of these electrodes has been developed for use in acute experiments. They can be easily and inexpensively made, and are adaptable to nerves of various diameters and situations. In constructing the electrodes, which are shown diagrammatically in Fig. 1, the following directions should be followed:

1. Attach eight 10 feet lengths of No. 32 enameled copper wire to the end of the shaft of a motor and revolve the shaft until the wire is evenly and closely twisted. The result is a very flexible conductor. This will be called the "flexible wire" (*F*). It should be cut into lengths of one foot.

2. Make loops (*A*) of soft silver wire (No. 27), about 2 to 3 mm. in diameter, to fit inside a No. 12 French catheter, a size generally useful. Leave about 1 cm. of the wire extending from the loop; to this extension wind and solder one end of the flexible wire (*F*) from which the enamel has been scraped. This soldering should be done before the loop is put into the catheter, otherwise the rubber will be damaged by the heat.

3. Cut a length of the catheter slightly greater than the desired distance between the electrodes and split it longitudinally. Make two holes (*B, B*) with a cutting needle, each one 2 mm. from the end of the tube and 2 mm. from the split edge. Carefully insert through the holes the silver loops and tie each of them in place with heavy silk (*C, C'*). Coat each wire, near its emergence from the catheter, with a good quality of rubber cement, and then slip over it a 3-inch

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piece of fine white stitch tubing (*D*, bore  $\frac{1}{32}$ , wall  $\frac{1}{64}$  inch) pushing it firmly against the catheter. In the figure this tubing is not shown covering the nearer wire.

4. After the two emergent wires have been thus prepared and covered with the stitch tubing, tie them together in the covered portion. Coat the tubing and the outer surface of the split piece of catheter with rubber cement. Beginning with the free edge of the piece of catheter, near *C'C'*, wrap over it a strip of finest dental rubber dam, and continue the wrapping around the stitch tubing. Thus the end connections are insulated and short circuits are rendered impossible.

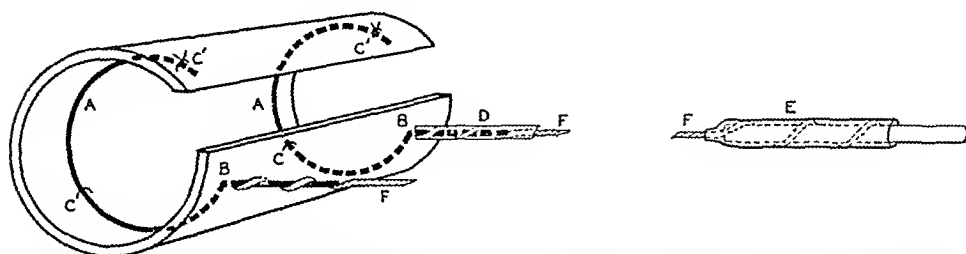


Fig. 1.—Diagram of shielded electrodes. *A, A*, silver wires; *B, B*, holes in the catheter; *C, C'*, silk ties to hold silver wire loops in place; *D*, rubber tube covering junction of silver wire and twisted flexible wire (*F*) (tube not represented over nearer junction); *E*, rubber tube covering junction of solid wire with twisted flexible wire (*F*).

5. Slip over each flexible wire (*F*) a 1-inch length of very small bore gray rubber tubing. To the scraped free ends of these wires solder a short piece of solid copper wire and then slide the rubber tubing back to cover the junction (*E*). This solid wire will serve as a connection to an induction coil.

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## TECHNICAL FACTORS INFLUENCING THE WELTMANN SERUM COAGULATION REACTION\*

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IN 1930 Weltmann described a serum coagulation reaction taking place in dilute calcium chloride solution, which he used as the basis of a nonspecific laboratory test for the presence of certain general pathologic processes. The test has been accepted widely in Europe and to an increasing extent in this country. There have been numerous reports of its value in following the course and therapy of various diseases, including rheumatic fever. Comparisons between the serum coagulation reaction and the sedimentation rate as guides to the treatment of the rheumatic fever patient have been made, and it was such a problem that first attracted our attention. While performing many Weltmann tests certain technical sources of error were found, and since these have not been mentioned previously, we wish to report them, together with means for their elimination.

The method of testing serum coagulability has been well described by Weltmann<sup>1</sup> and Tenfl,<sup>2</sup> and their directions were followed in performing and interpreting the reactions. Accordingly, we made up a stock solution of calcium chloride anhydrous, reagent Merck, a form of the salt containing at least 96 per cent calcium chloride. This solution was diluted further with distilled water to the concentrations necessary for the reaction. We then tested several normal sera with these dilutions but found that instead of a normal coagulation band (C. B.) of 6 or 6.5, they all gave bands of 8 and 9, or even 10. All workers agree that a coagulation band greater than 6.5 or rarely 7 indicates the presence of a proliferative or fibrotic process, while one less than 6 indicates an inflammatory or necrotic lesion. Triple distillation of the water failed to correct this, and in order to have less calcium chloride in our solutions we deviated from the directions by using calcium chloride dihydrate, USP XI, which contained 75.49 per cent of the anhydrous salt. Again we found the coagulation band of normal persons to be 7 or 8, and occasionally 9. In order to have even weaker solutions we then employed calcium chloride hexahydrate, reagent Merck, as described in a later paper by Weltmann and Sieder,<sup>3</sup> and a recent one by Levinson and Klein.<sup>4</sup> Of this preparation only 50.66 per cent is the pure salt. Even these weaker solutions produced coagulation bands of 7, 8, and 9 when normal sera were used. We never secured a normal coagulation band of 6 with any of these calcium chloride solutions.

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Testing the acidity of the electrolytic solutions by the colorimetric method, we found them to be between pH 6.3 and 6.5, which was the same as that of our distilled water. Because Weltmann<sup>1</sup> had said that his solutions were alkaline, and Levinson and Klein<sup>4</sup> had found theirs to be between pH 7.5 and 7.73, we adjusted ours to 7.6, with 0.1 normal sodium hydroxide. This was done by adjusting the distilled water to pH 7.6 prior to preparing the solutions to avoid their subsequent dilution. This was not a significant addition of cations, since Weltmann<sup>1</sup> found that the monovalent sodium did not react in the same way as the bivalent calcium. With our solutions adjusted in this way we tested a large number of normal sera and found each of them to have a coagulation band of 6 or 6.5.

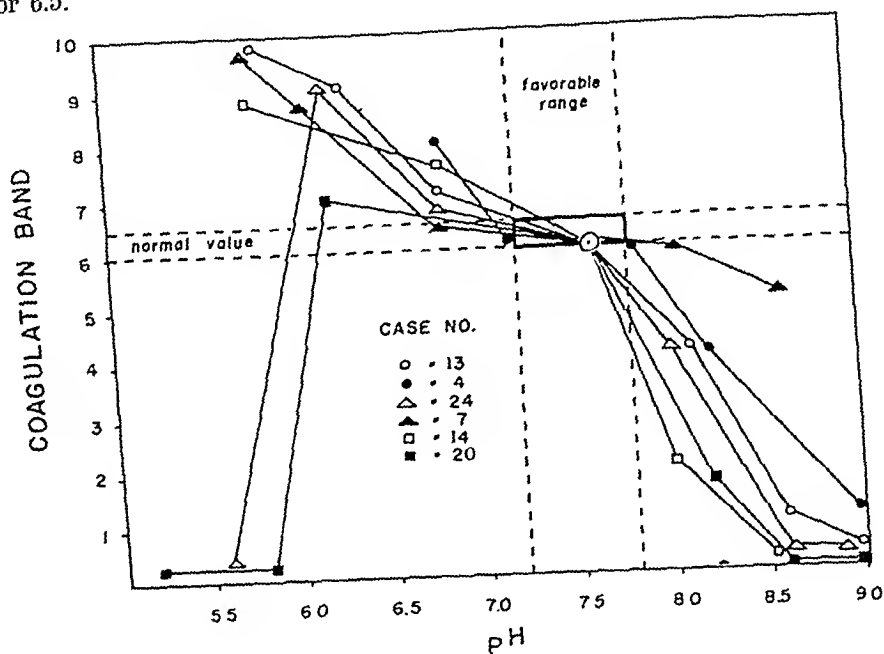


Fig. 1.—Behavior of coagulation bands of six normal sera at different pH values.

Accordingly, we studied in more detail the influence of the pH upon the coagulation of the serum. We found that as the solutions became more acid, the coagulation band was higher or shifted to the right; and as they became more alkaline, it was decreased or shifted to the left. When this phenomena was examined more closely, we found all normal sera reacted similarly in the pH range from 7.2 to 7.8, by giving a coagulation band of 6, or rarely 6.5. In more acid or more alkaline calcium chloride solutions normal sera did not always give the same coagulation band; for example, at pH 8.6 normal sera had coagulation bands from 0 to 3, and at pH 5.6 most coagulation bands ranged from 8 to 10. In some sera, however, the coagulation band would first shift to the right as the testing solutions became more acid; but when a pH of about 5.8 or less was reached, the coagulation band became 0. In these cases no observable change took place in the tubes, not even a clouding of the fluid. This led us to believe there probably had been some change in the coagulable protein. These effects of the pH were

found regardless of the strength of the calcium chloride salt used to prepare our testing solutions. For instance, with the dihydrate of the salt, a pH of 8.4 to 9.0 was necessary before normal serum gave a coagulation band of 6, and in less alkaline solutions the band was higher. Fig. 1 records the coagulation band of 6 normal sera tested with calcium chloride solutions of different acidity and alkalinity. Many others tested gave similar results.

The only references to the hydrogen-ion concentration in connection with the Weltmann test, which we could find, were those of Weltmann,<sup>1</sup> Levinson and Klein,<sup>4</sup> and Dees.<sup>5</sup> Weltmann<sup>1</sup> said that his solutions were alkaline; this may have been due to the alkalinity of the water in Vienna, a fact known to one of us (F. S.) during previous work. Levinson and Klein<sup>4</sup> mentioned the pH of their solutions but believed it had no effect on the coagulation band. In the same paper they report the influence of the serum pH on the reaction, pointing out that a decrease of the pH caused the coagulation band to shift to the right, while an increase caused the reverse to take place. This fact became more evident when their studies were repeated on the same patient. Despite this, they believed that there was no essential relationship between the pH and the coagulation band. Since we found the alkalinity of the testing solutions influenced the coagulation band, we felt that because the serum was diluted fifty times by these solutions, the pH of the mixture would be more nearly that of the larger volume of fluid. In other words, the pH of 5 c.c. of these solutions was not changed enough by the addition of 0.1 c.c. of serum to influence the reaction.

TABLE I

|    | <i>Amount CaCl<sub>2</sub><br/>Solution<br/>c.c.</i> | <i>pH CaCl<sub>2</sub><br/>Solution</i> | <i>Amount Serum<br/>c.c.</i>             | <i>pH Serum</i>          | <i>pH Mixture</i> |
|----|--|---|--|--------------------------|-------------------|
| 1. | 2.5  | 9.0                                     | 0.05                                     | 7.6                      | 8.6               |
| 2. | 2.5  | 8.6                                     | 0.05                                     | 7.6                      | 8.4               |
| 3. | 2.5  | 8.2                                     | 0.05                                     | 7.6                      | 7.9               |
| 4. | 2.5  | 7.6                                     | 0.05                                     | 7.6                      | 7.6               |
| 5. | 2.5  | 6.2                                     | 0.05                                     | 7.6                      | 6.8               |
| 6. | 2.5  | 5.8                                     | 0.05                                     | 7.6                      | 6.3               |
| 7. | 2.5  | 5.2                                     | 0.05                                     | 7.6                      | 5.8               |
|    | <i>Amount CaCl<sub>2</sub><br/>Solution<br/>c.c.</i> | <i>pH CaCl<sub>2</sub><br/>Solution</i> | <i>Distilled H<sub>2</sub>O<br/>c.c.</i> | <i>pH H<sub>2</sub>O</i> | <i>pH Mixture</i> |
| 1. | 5  | 8.4                                     | 0.1                                      | 7.6                      | 8.3               |
| 2. | 5  | 8.0                                     | 0.1                                      | 7.6                      | 7.9               |
| 3. | 5  | 7.6                                     | 0.1                                      | 7.6                      | 7.6               |
| 4. | 5  | 7.0                                     | 0.1                                      | 7.6                      | 7.1               |
| 5. | 5  | 6.7                                     | 0.1                                      | 7.6                      | 6.8               |
| 6. | 5  | 6.1                                     | 0.1                                      | 7.6                      | 6.2               |
| 7. | 5  | 5.6                                     | 0.1                                      | 7.6                      | 5.8               |

Dees<sup>5</sup> found that the pH of the calcium chloride solutions was variable and ranged between 5.25 and 6.30, but that when the serum was added the pH of the mixture became 7.2 to 7.4 in every case. She concluded that this was due to the buffering action of the serum, and that, for this reason, the acidity of the testing solutions was unimportant. In an attempt to repeat this, we adjusted the pH of our solutions and tested the influence of the serum on them at the various adjusted values, but failed to secure a strong buffering effect. That

there is slight buffering action is shown when water of the pH of the serum is added to the testing solutions in the same amount as serum, but this is not strong enough to bring the mixture to the favorable range if the solutions are very acid or alkaline. Many sera were tested in this way, and examples of our findings are shown in tabular form in Table I.

An incidental finding was that if our solutions were exposed to the air for any length of time before the test was made they became more acid, and consequently the bands shifted to the right. This was probably because of absorption of carbon dioxide from the atmosphere. Probably for the same reason the reagents became more acid daily, and, for this reason, the pH was tested and if necessary readjusted each time they were used. Because of this change we found it advisable to test a serum of known coagulation band when pathologic sera were tested, as well as whenever fresh solutions were made. This control need not be human serum but may be normal rabbit or sheep serum, which usually have a coagulation band of 6, but occasionally 5 or 7. In this connection it should be mentioned that Hennes and Kemen<sup>6</sup> advise making up fresh solutions every time the test is performed. This may have been due to the same unfavorable factors that influenced our reactions.

TABLE II

| <i>Material</i>                       | <i>Coagulation Band</i> |
|---------------------------------------|-------------------------|
| Normal beaten rabbit plasma           | 5                       |
| Normal beaten rabbit plasma + oxalate | 5                       |
| Normal human serum                    | 6                       |
| Normal human serum + oxalate          | 6                       |

Weltmann,<sup>1</sup> Teuff,<sup>2</sup> and Kaiser<sup>7</sup> always used fresh serum, and Levinson<sup>8</sup> said it was best to use fresh serum, but that it might be stored for as long as four days in the cold. We found that serum which was kept sterile and refrigerated gave the same coagulation band weekly for four and five weeks' time. Knowledge of this was valuable if for any reason we wanted to retest a serum.

We found that the reading of the test should be made immediately after the tubes were removed from the boiling water bath. If they were allowed to stand for any length of time, tubes which were densely cloudy without coagulation when removed from the bath, later showed a precipitate which might have been confused with coagulation. Hence tubes which should have been called negative would then be read falsely as positive, the coagulation band would be shifted to the right, and be greater than if the tubes were read at once.

Weltmann and Medvei<sup>9</sup> found that when they tested plasma of blood defibrinated by agitation with glass beads, the coagulation band was the same as that of the serum. In our experience plasma of blood treated in this way had a coagulation band that was shifted one or two tubes to the right of the serum value. It must be considered, however, that plasma treated in this way is slightly hemolytic, even though the agitation with the beads is done as carefully and as gently as possible. It is generally known that hemolysis increases the coagulation band. Plasma of blood treated with a mixture of potassium and ammonium oxalate usually had a coagulation band one or two less than the serum. Occa-

sionally it was the same as the serum, and very rarely it was slightly higher. That this was not due to the addition of oxalate (the mixture which has been used contains 3 Gm. of ammonium oxalate and 2 Gm. of potassium oxalate in 100 c.c. of distilled water; 0.08 c.c. has been used for 2 c.c. of serum) could be seen when oxalate was added to serum or beaten plasma, and the mixture was tested one hour later showing no change in the coagulation band (Table II).

#### SUMMARY AND CONCLUSIONS

The importance of using the hexahydrate of calcium chloride for solutions employed in the Weltmann test has been re-emphasized. The pH of these solutions was found to be an important factor influencing serum coagulation, and the favorable range for this coagulation is between pH 7.2 and 7.8, with an optimum value of pH 7.6. Since the buffering action of the serum used in the test is not great enough to secure favorable values, the reagents must be adjusted to the suggested range.

If such adjustment is made, the pH of the solutions should be determined every time before use, and a control serum of known value should be tested with unknown pathologic ones. This may be the serum of a suitable laboratory animal.

Serum tested need not be fresh if it has been kept sterile and refrigerated after collection.

The test should be read immediately after the tubes are removed from the boiling water bath to avoid a false positive interpretation.

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## AUSCULTATORY BLOOD PRESSURE METHODS FOR DOGS\*

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THE best blood pressure method accepted today for hypertension experiments in dogs seems to be that of subcutaneous arterial puncture,<sup>1</sup> which gives accurate pressures with little disturbance of the animal, and in expert hands permits fairly frequent repetition. However, when it is desired to make numerous daily estimations continued during many days or weeks, some indirect method is necessary. Aside from the antiquated van Leersum carotid loop, the choice is between oscillography, palpation, and auscultation. The need for comprehensive serial pressure readings prompted me<sup>2</sup> to devise the first auscultatory method for dogs as one of the preparatory steps for the contemplated renal-vascular research in 1923. The consensus of trials by various workers<sup>3</sup> has credited it with a reasonable degree of accuracy. Assuming, for argument, that errors may occur even greater than those reported, possibly to the extent of 10 or 15 mm. deviation from the true intra-arterial pressure, the general conclusion remains that direct methods may be used for precise acute experiments or for occasional checks on the indirect readings. For chronic experiments the frequently repeated readings by the convenient auscultatory method give a better picture of the general condition than a smaller number of technically exact determinations, which are subject to the far greater variations in the animal from hour to hour or from day to day. The reasons which make auscultation the preferred method for man may hold also for the dog.

Valuable criticisms and suggestions were made by Ferris and Hynes<sup>4</sup> in 1931. Upon finding an opportunity to resume this work recently, I have attempted a further improvement, which is illustrated in Fig. 1. The dog with very little training learns to lie quietly on his right side, and the pressure is taken in the left thigh. The hair is preferably, but not necessarily, clipped. The adapter, molded in hot water from dental gutta percha, is slightly curved to fit the posterior margin of the thigh. This appears to be an advantageous innovation as compared with former devices fitted to the external surface, because there is so little variation in the posterior margin that the one simple adapter will serve for most kinds and sizes of dogs. The adapter is thin above and thick below to compensate for the taper of the thigh. The surface is slightly roughened, and there are flanges above and below between which a 4 inch cuff can be held securely. Instead of a harness attachment to the body, a cord passes to a weight hanging over the edge of the table, heavy enough to assure that the adapter and cuff cannot slip downward.

Readings on the femoral artery are taken by means of a stethoscope with a flat bell slipped slightly under the lower margin of the cuff. There is a

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Fig. 1.—Adapter in position on posterior margin of left thigh, ready for application of cuff.



Fig. 2.—Dog lying on right side; left foreleg grasped with forefinger above olecranon; cuff applied above it.

possible advantage if the thick lower edge of the adapter spares the stethoscope slightly from too tight pressure under the cuff. If the bell piece is inserted too high, fictitiously high readings are obtained, just as when too narrow a cuff is used. Some practice is needed for the best wrapping of the cuff and accuracy of readings. Fairly large dogs are preferred, but readings have been obtained in Scotch and fox terriers. The feasibility of smaller equipment for still smaller dogs has not been investigated.

Sounds are audible in surprisingly small arteries, and I have confirmed the observation of Coreoran and Page<sup>5</sup> that in some large dogs readings are possible in the dorsalis pedis with a 2 inch cuff above the ankle. But, since attempts have been made for several generations to find a satisfactory method for the cylindrical foreleg of the dog, it was a surprise to learn finally that brachial artery readings are practically as simple and as distinct as in man. As shown in Fig. 2, the animal's left elbow is held straight by the left hand with the forefinger keeping a continuous grip above the olecranon. A 2 inch cuff is applied around the humerus above this forefinger. The cuff is thus kept high enough until the stethoscope is applied below (outside) it, at or slightly above the elbow joint. The difference from the femoral readings is similar to that between the human arm and thigh. Both systolic and diastolic sounds are usually so sharp and clear that this method may be favored over the more complicated femoral method. The systolic fluctuations are not as wide as in the femoral. In both, the diastolic may resemble that in man, or there may be either low or high readings of which the cause or significance is unknown. The method for both limbs, however, is quick and easy, and each can serve as a check on the other.\*

Shivering, panting, etc. may interfere seriously. Satisfactory sounds may be unobtainable in some states of anesthesia, hypotension, or weakness. The method, therefore, is not adapted to all types of experiments. For our hypertension studies, however, the numerous convenient combinations of systolic and diastolic, femoral and brachial readings have appeared more valuable than occasional mean pressures in a single artery.

#### SUMMARY

Convenient auscultatory methods are described for estimations of femoral and brachial blood pressures in dogs. Some advantages and limitations for different types of experiments are indicated.

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## THE LAUGHLIN TEST FOR SYPHILIS\*

### AN EVALUATION BASED ON COMPARISON WITH 3,100 HINTON TESTS

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IN 1935 Laughlen<sup>1</sup> described a flocculation test for syphilis which employs a special modification of the Kahn antigen. Among the advantages claimed for this test were simplicity of performance, speed, accuracy, and ease of reading the results. Essentially, the antigen for the Laughlen test is prepared from the Kahn antigen by the addition of cholesterol, scarlet R dye, and balsam. The dye, which is insoluble in water, colors the lipid particles of the antigen but does not color the liquid in which they are suspended. Thus, when flocculation occurs after mixing a drop of antigen with a drop of the serum to be tested on a glass slide, the clumped particles become visible to the naked eye and the reaction is, theoretically, easy to read. The directions for the preparation of the Laughlen antigen are outlined in the original report. Preparation of the antigen is somewhat complicated and considerable experience is necessary to adjust it to the proper sensitivity. Since 1937, however, a uniform and stable "Laughlen reagent," in its inactive form, has been produced by the Lederle Laboratories, and the antigen for the tests reported here was obtained from that source. Activated antigen for performing the test is prepared from the inactive material by the addition of 10 per cent sodium chloride. The exact amount of 10 per cent sodium chloride necessary to activate 1 c.c. is marked on each lot of antigen. Inactive antigen is stable for approximately six weeks, but the activated Laughlen reagent will keep for seven days only, after which it becomes super-sensitive. The antigen must be activated twenty-four hours prior to its use; it has been our practice to activate antigen once a week in a quantity sufficient to last seven days so that it is always available for use. Ordinarily, 1 c.c. of antigen suffices for about 75 tests.

In his first communication Laughlen,<sup>1</sup> reporting on 400 routine blood specimens, found that his test showed 99 per cent agreement with the Kahn test, and 98 per cent agreement with the Wassermann test. These findings were

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confirmed in general by Robinson and Stroud,<sup>2</sup> and Price.<sup>3</sup> The former investigators, in 1,000 routine tests on hospital patients, found 97 per cent agreement with the Kahn test and 93 per cent agreement with the Wassermann test. Later, Laughlen<sup>4</sup> studied more than 5,000 specimens according to his method and reported an agreement of 98 per cent with the Wassermann test, and 99.4 per cent with the Kahn test.

Originally, Laughlen<sup>4</sup> stated that his test could be performed successfully on spinal fluid as well as on fresh noninactivated blood samples. It is now generally agreed, however, that this is not so, and the pamphlet which accompanies the Lederle "Laughlen reagent" at present warns against using this test on unheated blood samples and spinal fluid. Furthermore, all the above-mentioned investigators,<sup>1-4</sup> recommended the Laughlen test for use in the doctor's office by physicians, and even by those who had no special training in serology. These unfortunate statements, however, have been retracted, and in recent months there have been no further exaggerated claims for the Laughlen test.

Since 1938 a number of reports on the Laughlen reaction have appeared in the literature. Several investigators have found the Laughlen test to be satisfactory under laboratory conditions, while other workers have thoroughly condemned the method. Muether and Greutter,<sup>5</sup> after studying 1,000 blood specimens, concluded that the Laughlen test "is somewhat less accurate" than the Kahn test but "compares fairly well" with the Kline test. Dienst and Sander-son<sup>6</sup> examined the blood sera of 276 known syphilitic patients, who had positive clinical manifestations of the disease, and found that the Laughlen test had a sensitivity of 96.3 per cent compared to 93.4 per cent for the Kahn test, and 77.8 per cent for the Kolmer-Wassermann test. At the same time these authors tested 687 presumably negative sera and found only one false positive reaction. Breazeale, Greene, and Harding<sup>7</sup> performed the Kahn, Eagle, Ide, Kline, and Laughlen tests on 1,000 sera and showed an agreement between the tests of 98.4 to 99.5 per cent. Craig and Callaway<sup>8</sup> examined the unheated sera from 1,000 patients and found that the Laughlen test agreed with the Wassermann test in 91.8 per cent of the cases. With the Kahn test, however, a 99.8 per cent agreement was obtained. In 1939 a comprehensive study of the Laughlen test was published by Beck.<sup>9</sup> She found, in comparing the Laughlen test with 1,000 Kline tests, 1,100 Kolmer-Wassermann tests, and 100 Kahn tests, that the Laughlen reaction was just as specific (99.3 per cent) and somewhat more sensitive (77 per cent) than the other tests. Price<sup>10</sup> reported that the Kahn and Laughlen tests detect treated syphilitic persons better than the Wassermann test. He also found that the Laughlen and Kahn tests remain positive longer than the Wassermann test in cases of treated syphilis. Recently, another valuable survey of the Laughlen test was completed by Lever and Massie.<sup>11</sup> They performed Laughlen tests on the sera of more than 2,200 persons and compared their findings with the results of the Hinton and Wassermann tests. They found that in 442 presumably syphilitic patients the Laughlen test had a sensitivity of 75.6 per cent compared with 97.5 per cent for the Hinton test, and 42.8 per cent for the Wassermann test. In 1,778 nonsyphilitic patients these workers found only 0.7 per cent false positive Laughlen reactions. Lever and Massie

also reported that the Laughlen test is less sensitive but more specific than the rapid Hinton test, and less specific but more sensitive than the Kahn test. Taschner<sup>12</sup> compared the results of the Kline, Kahn, and Laughlen tests on 1,500 blood samples and found that the specificity of the Kahn and Kline tests was approximately 99.6 per cent, while that of the Laughlen test was 98.6 per cent. He, therefore, concluded that the Laughlen reaction is somewhat less specific than the Kahn and Kline tests.

On the other hand, a number of investigators have been unable to confirm the above-mentioned favorable findings. Usher<sup>13</sup> reported that the specificity of the Laughlen test is considerably less than that of the Eagle flocculation test. Furthermore, Usher found that when the sensitivity of the Laughlen reagent is reduced sufficiently to eliminate false positive reactions, it fails in a significant number of instances to detect sera giving a positive reaction with the Eagle test. Rein and Hazay<sup>14</sup> found the Laughlen reaction less specific and less sensitive than the Kline test. Flood and Mayer<sup>15</sup> examined 314 nonsyphilitic blood specimens and obtained four false positive reactions with the Laughlen test, whereas the Kahn, Kline, and Kolmer-Wassermann tests each gave three false positive results. On the basis of this small series, Flood and Mayer came to the statistically doubtful conclusion that the Laughlen test is less specific than the other three. These authors also found that the Laughlen test becomes negative earlier than the Kline, Kahn, and Kolmer-Wassermann tests during antisyphilitic treatment, and that the Laughlen test detects less early syphilis and less untreated syphilis than the other three tests. More recently, Moore<sup>16</sup> compared the Laughlen test with the Kolmer-Wassermann and Kahn tests and found the Laughlen reaction to be too erroneous to justify recommendation. Chnrg and Sobel<sup>17</sup> attempted to evaluate the Laughlen test by studying the reaction under conditions comparable to those in a physician's office or in a small laboratory. These authors compared their results with more than 2,000 routine Wassermann tests performed on the same specimens by the Serologie Laboratory of the City of New York. They found the Wassermann test to be more sensitive and more specific than the Laughlen test. In addition to these divergent results, editorial comments<sup>18, 19</sup> have appeared which warn against the use of the Laughlen reaction by untrained persons.

Thus, there seems to be no general agreement in the literature concerning the value and effectiveness of the Laughlen test. This is unfortunate, since a rapid, accurate, and easily performed test for syphilis would obviously find many uses at the present time. Such a test, if it were sufficiently sensitive and specific, would be valuable in emergency transfusions under both war-time and peacetime conditions, in certain preoperative situations, and for the legally required premarital serologic tests now common in many states. In the light of these considerations, and because of the conflicting reports in the literature, it was felt that it might prove useful to re-evaluate the Laughlen test by comparing it with the Hinton test in a large series of cases. The latter test, as it is performed in Hinton's laboratory, is undoubtedly one of the best flocculation tests for syphilis available at the present time.<sup>20, 21</sup> For the purpose of this study, 3,100 sera furnished by the Massachusetts State Laboratory were tested by Laughlen's method over a period of five months, and the results were compared with those

obtained in that laboratory by the Hinton reaction. Of these, 305 specimens were from proved syphilitic patients, almost all of whom had undergone various amounts and kinds of treatment. A total of 1,094 blood samples were from persons who had no clinical evidences or history of syphilis, and these are designated as "presumably negative." The remaining 1,701 sera were from persons whose medical history is unknown so far as syphilitic infection is concerned.

## RESULTS

Of the 3,100 inactivated blood sera tested in this series, the results of the Laughlin test agreed with those of the Hinton test in 2,873 instances—an absolute agreement of 92.8 per cent. Of these, 609 were positive agreements, 2,256 were negative agreements, and 8 gave doubtful readings in both tests. Total disagreements numbered 227. These are analyzed in Table I.

TABLE I

227 CASES IN WHICH THE LAUGHLIN AND HINTON TESTS DISAGREED

| Laughlin | +  | 0  | +  | ?  | 0  | ?  |
|----------|----|----|----|----|----|----|
| Hinton   | 0  | +  | ?  | +  | ?  | 0  |
|          | 64 | 70 | 21 | 13 | 22 | 37 |

Such data are not entirely satisfactory, since the actual sensitivity and specificity of the Laughlin test remain undetermined. There were 305 patients in this series, however, who had clinical manifestations of syphilis and whose blood sera showed a positive Hinton test. All but one of these had received some form of antisymphilitic treatment at some stage of the disease, and 198, or 65 per cent, were patients with neurosyphilis. In this group of 305 inactivated syphilitic specimens the Laughlin test gave 247 positive, 56 negative, and two doubtful readings, or a sensitivity of 81 per cent. This figure approximates the results obtained by Beek,<sup>9</sup> and Lever and Massie,<sup>11</sup> but falls far short of the sensitivity quoted by Dienst and Sanderson.<sup>6</sup>

To determine the specificity of the Laughlin reagent there were available 1,094 patients who had never had clinical manifestations or history of syphilis, and whose blood specimens yielded a negative Hinton test. In this series of presumably nonsymphilitic inactivated blood samples, the Laughlin test showed 1,077 negative, 12 positive, and five doubtful reactions, or a specificity of 98.4 per cent. This specificity is somewhat less than that quoted for the Laughlin test by other investigators,<sup>6, 9, 11, 15</sup> and no obvious explanation for this could be found. It should be mentioned, however, that two false positive results were from patients who are now known to have had active tuberculosis at the time the specimens were obtained. Parran and Emerson<sup>22</sup> have reported that false doubtful and false positive serologic tests are often encountered in persons with active tuberculosis.

Recently, Kirk and Bennett<sup>23</sup> described a rapid technique for testing small amounts of capillary blood with the Laughlin reagent. This method employs *noninactivated* blood samples, and although it has been generally conceded that the Laughlin reaction is thoroughly unreliable when used to test *noninactivated* sera, these authors found that the Laughlin test, performed by their method,

yielded entirely satisfactory results. In an effort to repeat the work of Kirk and Bennett, 577 blood samples were tested by the Laughlen reaction, using both heated and unheated sera. The results were then compared with those obtained with the Hinton test in the Massachusetts State Laboratory. In this series, when inactivated sera were used, the Laughlen test agreed with the Hinton test in 529, or 91.6 per cent, of the cases. Of these, 52 were positive agreements, 476 were negative agreements, and in one instance both tests gave doubtful results. This total absolute agreement does not differ significantly from that obtained in the entire series of 3,100 tests. There were 48 disagreements. These are set forth in Table II.

TABLE II

48 INACTIVATED BLOOD SERA WHICH YIELDED DIVERGENT LAUGHLEN AND HINTON TESTS

| Laughlen<br>Hinton | +  | 0  | + | ? | 0 | ? |
|--------------------|----|----|---|---|---|---|
|                    | 0  | -  | ? | - | ? | 0 |
|                    | 12 | 20 | 3 | 2 | 4 | 7 |

On the other hand, when the sera in this group were Laughlen tested without previous inactivation, the results agreed with the Hinton test in only 492 instances—an agreement of 85.2 per cent. There were 34 positive, 456 negative, and two doubtful agreements. The 85 disagreements are analyzed in Table III.

TABLE III

85 NONINACTIVATED BLOOD SERA WHICH YIELDED DIVERGENT LAUGHLEN AND HINTON TESTS

| Laughlen<br>Hinton | +  | 0  | + | ? | 0 | ?  |
|--------------------|----|----|---|---|---|----|
|                    | 0  | -  | ? | - | ? | 0  |
|                    | 23 | 38 | 3 | 2 | 3 | 16 |

These results indicate that the absolute agreement between the Laughlen and Hinton tests is considerably less when the Laughlen reaction is performed with unheated blood sera. Heretofore it has been reported<sup>17</sup> that it is the sensitivity of the Laughlen test which is decreased when noninactivated blood samples are employed. From Tables II and III, however, it seems that both the specificity and the sensitivity of the Laughlen reaction are seriously affected when unheated blood sera are used for the test. In addition, the difficulty of reading the Laughlen results is markedly increased when the test is carried out with noninactivated blood. This is suggested by the increased number of doubtful Laughlen readings in Table III.

In an attempt to determine further the effect of noninactivated blood sera on the specificity and sensitivity of the Laughlen test, blood samples were obtained from 31 known syphilitic patients and 350 presumably nonsyphilitic patients. These sera were then Laughlen tested before and after inactivation by heating at 56° C. for thirty minutes. The known syphilitic samples were Hinton tests. Presumably nonsyphilitic sera were from persons who showed no clinical evidences of syphilis and whose inactivated blood sera gave a negative Hinton test. In the small group of 31 known syphilitic blood samples the Laughlen test, performed on inactivated sera, showed 26 positive, five negative,



and no doubtful readings, or a sensitivity of 80.7 per cent. When the same syphilitic sera were Laughlin tested without previous inactivation, however, there were 19 positive, one doubtful, and 11 negative results, a sensitivity of only 61.2 per cent. In the series of 350 presumably nonsyphilitic blood specimens, the Laughlin test, after inactivation of the sera, yielded 343 negative, four positive, and three doubtful results for a specificity of 98 per cent. When the identical blood samples were tested by the Laughlin reaction, using unheated sera, there were 328 negative, nine positive, and thirteen doubtful readings for a specificity of 92.7 per cent.

The foregoing results reaffirm the conclusion that an attempt to develop a more rapid Laughlin technique by using unheated blood sera for testing is dangerous. Not only are the sensitivity and specificity of the test diminished, but the reaction is more difficult to read accurately when noninactivated sera are used.

#### COMMENT

The Laughlin test, possibly because of the exaggerated claims originally made for it, has not attained widespread clinical application up to the present time. The test is rapid and not particularly difficult to interpret, providing the observer has had sufficient experience. The antigen, as it is now supplied, is uniformly stable, and the preparation of the active antigen from the inactive material is relatively simple. Although the antigen must be activated at least twenty-four hours before use and although the active antigen is stable for only seven days, it is easy to prepare a week's supply of the reagent at one time. Such considerations would seem to make the Laughlin test particularly valuable for emergencies and for use in smaller outlying or isolated institutions, where it may be inconvenient or too expensive to perform other recognized tests for syphilis. Despite any claims to the contrary, however, considerable experience is necessary to read the Laughlin test accurately. In addition, the Laughlin test cannot safely be used to test spinal fluid or noninactivated blood serum.

From the confusing reports found in the literature, one cannot decide whether or not the accuracy of the Laughlin test justifies its acceptance. In general, it may be said that most of the evaluations which merely compare the percentage agreement of the Laughlin test with other well-known tests for syphilis on routine blood samples, are essentially unenlightening. A much more significant evaluation of any test for syphilis can be obtained by testing a series of known syphilitic and known nonsyphilitic blood samples, and expressing the results directly in terms of sensitivity and specificity.

In this study the Laughlin test performed on inactivated blood sera had an indicated sensitivity of 81 per cent, and a specificity of 98.4 per cent. This compares with a 77 per cent sensitivity and 99.3 per cent specificity obtained by Beck,<sup>9</sup> and a 75.6 per cent sensitivity and 99.3 per cent specificity reported by Lever and Massie.<sup>11</sup> From these results it seems that the Laughlin test is considerably less sensitive than the Kline and Hinton tests and approximately as sensitive as the Kahn test.<sup>20</sup> The specificity reported in this series is definitely less than that found by Beck, and Lever and Massie. Ordinarily, an acceptable

serologic test for syphilis must maintain a specificity of at least 99 per cent—a figure which the Laughlen test did not attain in our hands.

By its nature the chief attraction of the Laughlen test probably lies in its ability to exclude quickly the possibility of syphilis in certain clinical emergencies and diagnostic dilemmas. But the fact that the Laughlen test has an apparent sensitivity of only 75 to 80 per cent seriously detracts from its value in just those situations in which it should be most useful. On the other hand, if the Laughlen antigen is used in a supersensitive state, or if the results of the test are read closely enough to detect 95 to 100 per cent of syphilitic sera, then the specificity of the reaction falls markedly.<sup>9</sup> Nevertheless, the accuracy of the relatively simple Laughlen test is such that it may find a useful role in emergency situations, although its value in this respect probably does not equal that of the rapid Hinton test.<sup>11</sup> Greer, Bracken, and Paul<sup>12</sup> have recently indicated that the Laughlen test is a satisfactory method for detection of syphilis in blood donors, provided the serum is inactivated before testing. These authors also emphasize that the Laughlen reaction should always be confirmed by methods of greater specificity before a final diagnosis of syphilis is made.

If, as Dieust and Sanderson report,<sup>6</sup> the Laughlen test is 96.3 per cent sensitive and 99.9 per cent specific, then it probably is the best test for syphilis now available. No other investigators, however, have obtained results approximating these. On the other hand, to condemn the specificity of the Laughlen test as Flood and Mayer<sup>13</sup> have done on the basis that it showed four false positives whereas the Kahn, Kline, and Kolmer tests yielded only three false positives in 314 cases, is statistically unreasonable. The truth of the situation probably lies somewhere between these extreme viewpoints.

#### CONCLUSIONS

The Laughlen test is rapid and, in general, is approximately as easy to read as most acceptable flocculation tests for syphilis.

Of a total of 3,100 inactivated blood sera tested in this series, the results of the Laughlen reaction agreed with those of the Hinton test in 92.8 per cent of the cases. The sensitivity of the Laughlen test in 305 syphilitic patients was 81 per cent, and its specificity was 98.4 per cent in 1,094 nonsyphilitic persons.

The Laughlen test is thoroughly unreliable when used to test noninactivated blood sera. Not only is the sensitivity of the test markedly reduced, but its specificity is adversely affected, and the results of the test are more difficult to read.

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## AUTOMATIC PIPETTE WASHER AND RINSER

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WE HAVE in this laboratory devised a pipette washer. This instrument, almost entirely automatic, requires less than three minutes of attention each day. By its use glassware can easily be made chemically clean. The instrument also saves some of the costs of cleaning fluid, water, and breakage. Since the full advantages of such an instrument may not be fully obvious, it may be of value to review, if only briefly, both the necessity for clean glassware and the methods by which chemical cleanliness may be obtained.

By definition glass is chemically clean when its surface is so free of extraneous matter that a film of water distributes itself over the surface of the glass in a layer, monomolecular in thickness. When a pipette is dirty, drop-

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lets, however small, remain attached to its surface. A dirty pipette will, therefore, not drain within its limits of error. In a micropipette the error may exceed 10 per cent. Since all chemistry, routine or research, is based upon quantitative analysis, it is absolutely necessary that all pipettes be chemically clean. So high a standard of cleanliness is not really difficult to achieve. All that is needed is a good cleaning fluid and an efficient rinsing system.

Chromic acid is the best cleaning fluid available. It is not, however, always made by the proper methods nor is it handled with the proper care. To make it cheaply and to handle it so that it may be efficient for as long as possible, the following technique should be used:

Five grams of technical potassium bichromate should be dissolved in a minimum amount of water. To this concentrated aqueous solution of potassium bichromate should be added one liter of technical sulfuric acid. Any amount may be made as long as the proportions given are followed. The final solution is dark brown, and in it may be seen the suspended red chromic anhydride crystals. The chromic anhydride is the oxidizing agent. When reduced, it turns to the green chromic oxide and is then useless for cleansing purposes and must, therefore, be discarded. The oxidizing capacity is destroyed by reducing agents, especially organic solvents as alcohol and acetone. All glassware should be rinsed before being placed in the chromic acid solution. The sulfuric acid is hygroscopic, and the solution will absorb atmospheric water unless the cylinder in which it is contained is closed to the atmosphere by a glass plate. Heat is produced when the acids are mixed. They may be mixed with safety if the cylinder is made of pyrex glass.

With the usual method of washing pipettes, each pipette is taken in turn from the acid solution. One end is connected to a suction apparatus, and the pipette is rinsed in running water. Since water is used for both the suction and the cleaning, and since each pipette is handled individually, the process is noisy, tedious, and wasteful. Some breakage is inevitable.

With the instrument described below, any number of pipettes can be cleaned, and yet no individual pipette is handled from the time it is soiled until it is again clean, dry, and ready for use. The system can run at night and, in all, requires less than three minutes of attention for any batch of pipettes. No breakage is possible during the cleansing process.

The complete apparatus consists of two glass cylinders, both made of pyrex and both of the same size; and a third smaller glass cylinder which sits upright in either of the other two. Since the smaller cylinder (marked B) is the most important part of the apparatus, it is described first.

During the working day cylinder B stands or lies on its side on the laboratory bench; as the pipettes are soiled, they are placed in it with their tips upward. The base of cylinder B is in the form of a hemisphere so that the mouth-piece of each pipette stands at a tangent. The passage of the water or cleansing fluid is completely free.

When it is necessary to clean the pipettes, cylinder B is lowered into cylinder A. Cylinder A is so constructed that there is an inlet of small caliber and a siphon outlet of large caliber. When the water is turned on, it fills both cylinders, its level rising until the bend of the top of the siphon is reached.

Both cylinders then empty, since the rate of outflow is faster than the inflow. That arm of the siphon which is inside cylinder A is grooved at its lower end so that when the water falls to this level, the air breaks the flow through the siphon. As soon as this occurs, both cylinders begin to fill again. Each filling and emptying requires between five and seven minutes so that it is possible to obtain ten or more washings per hour.

With the inlet of cylinder A attached to tap water, the pipettes are rinsed and drained, and the material so removed helps to preserve the cleaning fluid.

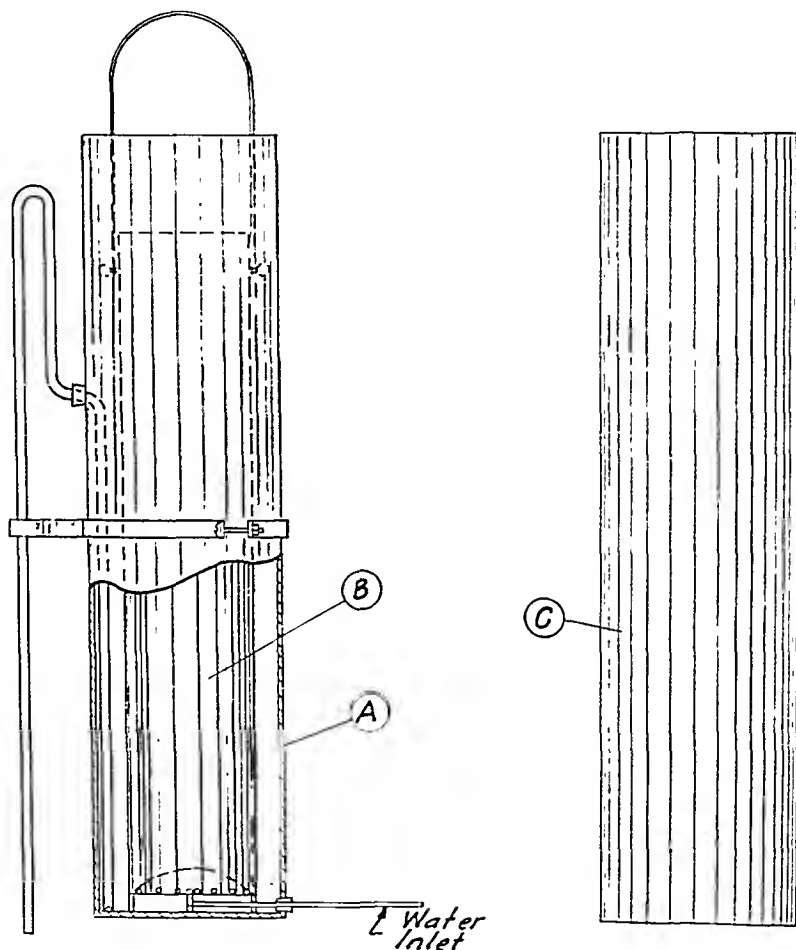


Fig. 1.

After three or more such flushings (the number is not of importance since the process is automatic) cylinder B is lifted free of cylinder A and allowed to drain, or else the siphon is permitted to empty both cylinders and the water is turned off. Cylinder B is then transferred to the chromic acid bath, cylinder C. The pipettes are usually permitted to stand in this acid for ten to twelve hours or overnight. When it is necessary to remove them, cylinder B is now raised by means of a lead handle, and the acid drains back into cylinder C. Cylinder B is now placed in cylinder A, the flow of tap water adjusted and the apparatus again permitted to fill and empty automatically. After the rinsing procedure

has continued for a sufficient time, the pipettes may be rinsed in distilled water by changing the connection at the inlet of cylinder A. A double connection, one for tap and the other for distilled water, may be used. Cylinder B may then be removed and placed in a drying oven. A glass plate may be used for covering cylinder B, which can then be permitted to remain on the laboratory bench. The pipettes will drain and dry, and, if time is not a factor, no gas oven is necessary. The height of the siphon can, of course, be adjusted so that short pipettes may be washed without the necessity of filling cylinder A to the top, saving, therefore, both tap and distilled water.

By the procedures outlined as used with the apparatus described, pipettes can be made chemically clean. The procedure requires only a simple technique which avoids breakage, saves time, and lowers laboratory expense, since it uses less tap water, distilled water, and cleansing fluid than any of the present methods. The pipettes rendered chemically clean by this method make quantitative analyses much more accurate.

75 BAY STATE ROAD

This apparatus is available through E. Machlett and Son, 220 East 23rd Street, New York, N. Y.

## CHEMICAL

### THE RELATIVE STABILITY AND POTENCY OF THROMBOPLASTINS FOR PROTHROMBIN TESTS\*

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IN OUR preliminary report<sup>1</sup> we stated that thromboplastin solutions prepared from dried rabbit brain are remarkably stable. This finding is contrary to Quick's<sup>2</sup> statement, for we have observed that saline solutions of dried rabbit brain retain their potency for as long as six months. However, the duration of the thromboplastic potency of these solutions varies decidedly according to the method employed in preparing the original brain powder used in making the suspensions. In order to test the relative potencies of thromboplastins, and to ascertain the duration of their activities, the dried brain powders were prepared according to the two most commonly used procedures, namely: (1) the acetone-dehydration method of Quick, and (2) the incubator-desiccation method of Magath.<sup>3</sup>

#### EXPERIMENTAL

This investigation, conducted over a period of six months following the initial preparation of one composite of rabbit brains, consisted of 14 series of observations at biweekly intervals (Table I). The potency of each thromboplastic reagent prepared throughout this study was ascertained by determining its prothrombin clotting time with normal human plasma. The technique of prothrombin time used in this report is that of Quick's venous plasma dilution method.<sup>4</sup> Since the technical details are of paramount importance in an investigation of this nature, each step of the experimental procedures will be described somewhat at length.

*Removal of the Brain.*—From 8 to 10 c.c. of air were injected into the hearts of 18 normal rabbits by ventricular punctures, after which within one minute the animals usually died from air embolism. In an attempt to provide as bloodless a field as possible in the brain area, the animals were suspended by their necks immediately after death for approximately fifteen minutes. The brain was then removed from each animal in the following manner:

A longitudinal scalp incision was made between the occipital and frontal regions, so that the scalp could be separated into the lateral flaps. Using a rongeur, a niche was made in the parietal region of the skull. The brain was exposed by carefully chipping away the cranial bone, the movement being in a forward and lateral direction until the olfactory lobes were brought into view,

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and at the same time the meninges were also removed. The same procedure was carried out in a backward direction from the parietal region to the foramen magnum, thereby exposing the brain from the olfactory lobes to the spinal cord. Care was taken not to disturb the brain at any time while the rongeur was in the cranial cavity.

A small, flexible spatula was inserted below the olfactory lobes; by pushing it backward to the cerebellum the encephalon was freed from the base of the skull, except for the attachment at the optic chiasma. By moving the animal's head upward and backward through a  $120^\circ$  arc, the brain was easily severed from the optic chiasma. The animal's head was held in this backward position to allow the brain to fall into a position that would reveal the foramen magnum as well as the cranial nerves. By moving the spatula still farther back, the cranial nerves were cut, followed by a transection of the spinal cord just below the foramen. The final step was to cut the meninges at the tentorium cerebelli so that the brain could be completely removed from the cranium by a final upward movement of the spatula.

TABLE I

QUANTITATIVE DATA ON THE PREPARATION OF THE THROMBOPLASTIC SOLUTIONS AND PLASMA

| SERIES | NO. PERIODS PER SERIES | EACH SOLUTION REQUIRED (C.C.) | EACH SOLUTION PREPARED (C.C.) | EACH BRAIN POWDER REQUIRED (GM.) | SALINE ADDED (C.C.) | TOTAL BRAIN POWDER REQUIRED (GM.) | PLASMA REQUIRED (C.C.) |
|--------|------------------------|-------------------------------|-------------------------------|----------------------------------|---------------------|-----------------------------------|------------------------|
| I      | 14                     | 5.6                           | 11.2                          | 0.56                             | 10.64               | 2.24                              | 1.6                    |
| II     | 13                     | 5.2                           | 10.4                          | 0.52                             | 9.88                | 2.08                              | 4.0                    |
| III    | 12                     | 4.8                           | 9.6                           | 0.48                             | 9.12                | 1.92                              | 5.6                    |
| IV     | 11                     | 4.4                           | 8.8                           | 0.44                             | 8.36                | 1.76                              | 7.2                    |
| V      | 10                     | 4.0                           | 8.0                           | 0.40                             | 7.60                | 1.60                              | 8.8                    |
| VI     | 9                      | 3.6                           | 7.2                           | 0.36                             | 6.84                | 1.44                              | 10.4                   |
| VII    | 8                      | 3.2                           | 6.4                           | 0.32                             | 6.08                | 1.28                              | 12.0                   |
| VIII   | 7                      | 2.8                           | 5.6                           | 0.28                             | 5.32                | 1.12                              | 13.6                   |
| IX     | 6                      | 2.4                           | 4.8                           | 0.24                             | 4.56                | 0.96                              | 15.2                   |
| X      | 5                      | 2.0                           | 4.0                           | 0.20                             | 3.80                | 0.80                              | 16.8                   |
| XI     | 4                      | 1.6                           | 3.2                           | 0.16                             | 3.04                | 0.64                              | 18.4                   |
| XII    | 3                      | 1.2                           | 3.0                           | 0.15                             | 2.85                | 0.60                              | 20.0                   |
| XIII   | 2                      | 0.8                           | 3.0                           | 0.15                             | 2.85                | 0.60                              | 21.6                   |
| XIV    | 1                      | 0.4                           | 3.0                           | 0.15                             | 2.85                | 0.60                              | 23.2                   |

*Preparation of the Brain Powder.*—After stripping the brains of the remaining fragments of the meninges and the discernible blood vessels, they were macerated to a smooth paste in a large mortar. This composite of fresh rabbit brains, weighing altogether 97 Gm., was equally divided into two portions. One portion was spread in a thin film over a glass plate, in accordance with the technique described by Magath,<sup>3</sup> and dried at  $37.5^\circ$  C. for sixteen hours. The dried brain paste was then removed from the glass by scraping it off in fine flakes with a small flexible spatula. The second portion was dehydrated with acetone and dried as recommended by Quick.<sup>2</sup>

The Magath and Quick thromboplastins were again divided into two equivalent portions; one part of each preparation was stored at  $5^\circ$  C. in separate tightly stoppered bottles and the other part was kept at room temperature in well-stoppered containers. From this original composite of fresh rabbit brain four types of thromboplastic material were thus available for investigation,



namely: (1) Quick's brain powder stored at 5° C.; (2) Quick's brain powder kept at room temperature; (3) Magath's brain powder stored at 5° C.; and (4) Magath's brain powder kept at room temperature.

*Preparation of the Thromboplastic Reagents.*—At fortnightly intervals adequate amounts of the four types of thromboplastic material for each series (Table II) were suspended in 19 parts of freshly prepared 0.85 per cent sodium chloride solution, thus making a 1:20 solution.\* To insure a uniform preparation of all the thromboplastic reagents throughout this investigation, the suspensions of dried rabbit brain were always made in 9 by 65 mm. test tubes (Kolmer serum tubes). After thorough mixing, the saline suspensions of thromboplastin were warmed for fifteen minutes at 45° C. and then were again thoroughly mixed and finally allowed to stand for sedimentation of the coarse particles. The milky supernatant liquid from each of the four types of brain powder was then transferred to stoppered vials. For one-half of the experiments it was necessary to use several Kolmer serum tubes in order to make enough of one sample of a single thromboplastic reagent for a given series. After the sedimentation of the coarse particles the supernatant liquids from one type of rabbit brain powder were pooled together in the same vial.

*Preparation of the Chemical Reagents.*—The chemical reagents required in the performance of the prothrombin clotting time determinations in the present investigation were prepared according to the following specifications:

(1) M/40 calcium chloride solution: 0.368 Gm. of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (Baker's special grade) dissolved in 100 c.c. of distilled water.

(2) Physiologic saline: 0.85 Gm. of Merek's reagent NaCl in 100 c.c. of distilled water.

(3) 1.34 per cent sodium oxalate solution: 1.34 Gm. of  $\text{Na}_2\text{C}_2\text{O}_4$  (Baker's analyzed grade) in 100 c.c. of distilled water.

*Collection of Plasma.*—For each series of experiments sufficient blood was drawn from patients with hypertension. If great difficulty was encountered in introducing the needle into the vein, the sample of blood was rejected. A freshly sterilized clean needle and syringe were used for each new venipuncture attempted at a different site. The blood was transferred without delay from the syringe into a tube containing 1.34 per cent sodium oxalate in such proportions as to furnish 1 part of anticoagulant to 9 parts of blood. After thorough mixing of the contents, the tube was centrifuged for fifteen minutes at 2,000 r.p.m. for complete separation of the plasma. Except for the 2 c.c. that was to be immediately used for the analysis, the stock supply of plasma was kept at 5° C. to prevent any possible diminution of its prothrombin activity, as well as any possible deterioration of the plasma itself. As more plasma was needed for the series of tests, 2 c.c. portions were removed from the stock supply.

*Assay of the Thromboplastic Material.*—The tests performed to assay the thromboplastic solutions thus prepared were carried out at 37.5° C.  $\pm$  1° C. One-fortieth molar calcium chloride solution was kept in a constant temperature water bath throughout the period of examination.

\*Experience with various dilutions of rabbit brain powder with normal saline has shown a proportion of 1:20 (Kelley and Bray<sup>2</sup>) to give the same prothrombin clotting time as that of 1:18 dilution (Quick).

TABLE  
PROTHROMBIN TIME IN SERONS  
(ONLY EVERY OTHER SERIES)

| DATE OF<br>ASSAY<br>PERIOD | SERIES I     |       | SERIES II    |       | SERIES IV    |       | SERIES VI    |      |
|----------------------------|--------------|-------|--------------|-------|--------------|-------|--------------|------|
| 1940<br>4/19               | Q5 (F-F)     | 18.0  |              |       |              |       |              |      |
|                            | Qr (F-F)     | 18.6  |              |       |              |       |              |      |
|                            | M5 (F-F)     | 27.2  |              |       |              |       |              |      |
|                            | Mr (F-F)     | 27.7  |              |       |              |       |              |      |
| 5/ 3                       | Q5 (14-14)   | 17.9  | Q5 (14-F)    | 18.6  | Qc           | 16.9  |              |      |
|                            | Qr (14-14)   | 17.6  | Qr (14-F)    | 18.4  |              |       |              |      |
|                            | M5 (14-14)   | 26.2  | M5 (14-F)    | 27.0  | Mc           | 24.7  |              |      |
|                            | Mr (14-14)   | 25.4  | Mr (14-F)    | 27.1  |              |       |              |      |
| 5/31                       | Q5 (42-42)   | 18.6  | Q5 (42-28)   | 19.2  | Q5 (42-F)    | 20.0  | Qc           | 18.0 |
|                            | Qr (42-42)   | 17.4  | Qr (42-28)   | 17.8  | Qr (42-F)    | 22.5  |              |      |
|                            | M5 (42-42)   | 21.3  | M5 (42-28)   | 27.7  | M5 (42-F)    | 28.9  | Mc           | 25.7 |
|                            | Mr (42-42)   | 24.6  | Mr (42-28)   | 24.4  | Mr (42-F)    | 30.4  |              |      |
| 6/28                       | Q5 (70-70)   | 17.5  | Q5 (70-56)   | 18.3  | Q5 (70-28)   | 19.7  | Q5 (70-F)    | 19.8 |
|                            | Qr (70-70)   | 16.1  | Qr (70-56)   | 17.0  | Qr (70-28)   | 19.9  | Qr (70-F)    | 23.8 |
|                            | M5 (70-70)   | 22.6  | M5 (70-56)   | 28.1  | M5 (70-28)   | 23.9  | M5 (70-F)    | 31.8 |
|                            | Mr (70-70)   | 24.8  | Mr (70-56)   | 25.4  | Mr (70-28)   | 26.8  | Mr (70-F)    | 27.4 |
| 7/26                       | Q5 (98-98)   | 20.6  | Q5 (98-84)   | 25.7  | Q5 (98-56)   | 20.2  | Q5 (98-28)   | 18.0 |
|                            | Qr (98-98)   | 20.4  | Qr (98-84)   | 20.9  | Qr (98-56)   | 27.3  | Qr (98-28)   | 22.9 |
|                            | M5 (98-98)   | 38.0  | M5 (98-84)   | 40.1  | M5 (98-56)   | 36.9  | M5 (98-28)   | 23.8 |
|                            | Mr (98-98)   | 31.0  | Mr (98-84)   | >40.0 | Mr (98-56)   | 33.5  | Mr (98-28)   | 25.1 |
| 8/23                       | Q5 (126-126) | 20.3  | Q5 (126-112) | 25.8  | Q5 (126-84)  | 22.8  | Q5 (126-56)  | 18.5 |
|                            | Qr (126-126) | 21.3  | Qr (126-112) | 20.1  | Qr (126-84)  | >40.0 | Qr (126-56)  | 25.0 |
|                            | M5 (126-126) | >40.0 | M5 (126-112) | >40.0 | M5 (126-84)  | >40.0 | M5 (126-56)  | 24.7 |
|                            | Mr (126-126) | >40.0 | Mr (126-112) | >40.0 | Mr (126-84)  | >40.0 | Mr (126-56)  | 27.5 |
| 9/20                       | Q5 (154-154) | 20.1  | Q5 (154-140) | 25.8  | Q5 (154-112) | 22.3  | Q5 (154-84)  | 17.8 |
|                            | Qr (154-154) | 20.2  | Qr (154-140) | 19.9  | Qr (154-112) | >40.0 | Qr (154-84)  | 25.3 |
|                            | M5 (154-154) | >40.0 | M5 (154-140) | >40.0 | M5 (154-112) | >40.0 | M5 (154-84)  | 26.5 |
|                            | Mr (154-154) | >40.0 | Mr (154-140) | >40.0 | Mr (154-112) | >40.0 | Mr (154-84)  | 29.9 |
| 10/18                      | Q5 (182-182) | 19.0  | Q5 (182-168) | 25.0  | Q5 (182-140) | 21.0  | Q5 (182-112) | 17.1 |
|                            | Qr (182-182) | 19.3  | Qr (182-168) | 19.5  | Qr (182-140) | >40.0 | Qr (182-112) | 26.5 |
|                            | M5 (182-182) | >40.0 | M5 (182-168) | >40.0 | M5 (182-140) | >40.0 | M5 (182-112) | 24.0 |
|                            | Mr (182-182) | >40.0 | Mr (182-168) | >40.0 | Mr (182-140) | >40.0 | Mr (182-112) | 26.5 |

Abbreviations: Q and M indicate Quick's and Magath's thromboplastins. 5, r, and c, immediately following Q and M designate the brain powder kept at 5° C., at room temperature.

In a duplicate setup 0.1 c.c. of plasma was introduced into each serum tube (9 by 65 mm.) containing 0.1 c.c. of physiologic saline, followed by 0.1 c.c. of the thromboplastin to be tested.

After permitting the thromboplastin-plasma mixture to stand at room temperature for fifteen minutes, the tubes were warmed in the 37.5° C. water bath for one minute before the actual test was performed for each tube so prepared. The final step was the addition of 0.1 c.c. of calcium chloride solution, and at the same instant the stop watch was started. The tube was kept in the bath for the first five seconds\* and was then held up against a source of light to insure good visibility of its contents. The tube was slowly inverted (about once in two

\*Preliminary tests have shown that the prothrombin time values obtained by this technique were identical with those obtained by keeping the tube fixed in another large tube containing water at 37.5° C. and slowly inverted.

II

OF AGING THROMBOPLASTINS  
AFTER SERIES II IS INCLUDED)

| SERIES VIII |      | SERIES X    |      | SERIES XII  |      | SERIES XIV |              |
|-------------|------|-------------|------|-------------|------|------------|--------------|
| Qc          |      | 16.5        |      |             |      |            |              |
| Mc          |      | 24.1        |      |             |      |            |              |
| Q5 (98-F)   | 19.1 | Qc          | 18.8 |             |      |            |              |
| Qr (98-F)   | 25.1 |             |      |             |      |            |              |
| M5 (98-F)   | 23.8 | Mc          | 21.0 |             |      |            |              |
| Mr (98-F)   | 24.3 |             |      |             |      |            |              |
| Q5 (126-28) | 19.1 | Q5 (126-F)  | 21.7 | Qc          | 17.2 |            |              |
| Qr (126-28) | 24.9 | Qr (126-F)  | 20.1 |             |      |            |              |
| M5 (126-28) | 25.0 | M5 (126-F)  | 23.2 | Mc          | 22.2 |            |              |
| Mr (126-28) | 25.0 | Mr (126-F)  | 23.8 |             |      |            |              |
| Q5 (154-56) | 19.6 | Q5 (154-28) | 27.2 | Q5 (154-F)  | 20.6 | Qc         | 18.2         |
| Qr (154-56) | 26.1 | Qr (154-28) | 22.2 | Qr (154-F)  | 33.5 |            |              |
| M5 (154-56) | 25.2 | M5 (154-28) | 25.4 | M5 (154-F)  | 28.5 | Mc         | 25.4         |
| Mr (154-56) | 27.7 | Mr (154-28) | 28.5 | Mr (154-F)  | 26.8 |            |              |
| Q5 (182-84) | 19.6 | Q5 (182-56) | 29.4 | Q5 (182-28) | 21.6 | Q5 (182-F) | 20.8 Qc 15.2 |
| Qr (182-84) | 26.6 | Qr (182-56) | 20.3 | Qr (182-28) | 32.0 | Qr (182-F) | 34.8         |
| M5 (182-84) | 22.3 | M5 (182-56) | 24.6 | M5 (182-28) | 24.0 | M5 (182-F) | 26.0 Mc 25.1 |
| Mr (182-84) | 24.2 | Mr (182-56) | 27.7 | Mr (182-28) | 27.2 | Mr (182-F) | 28.7         |

and the control. The figures enclosed in the parentheses represent the ages in number of days of the brain powders and of saline suspensions, respectively; F designates a fresh preparation. The sign > means greater than.

seconds) until the final reaction (clot formation) took place, the watch being simultaneously stopped. The results presented in Table II are averages of duplicate values which checked with each other within one second.

*Plan of Experiment.*—The prothrombin times obtained with fresh preparations of Magath's and Quick's thromboplastins with normal adult human plasma were noted first. The brain powders and the saline solutions were then stored in stoppered vials. All thromboplastin suspensions and part of each type of brain powder were kept at 5° C., the other part being stored at room temperature.

Two weeks later the activities of the original extracts, as well as those freshly prepared from another portion of the original composite of brain powder (by this time two weeks old), were tested on aliquots of one sample of normal plasma. Using this same sample of plasma, freshly made thromboplastin suspensions

from new Magath and Quick brain powders were tested for use as control values. This same plan of investigation was repeated at fortnightly intervals over a period of one hundred and eighty-two days (twenty-six weeks with thirteen bi-weekly testings).

For each experiment all the thromboplastic extracts on hand from the original composite of brain powder were tested and compared with a new solution of thromboplastin made from freshly prepared rabbit brain powder.

#### COMMENT

The experimental plan employed in the present investigation thus permitted the comparison of the stability of thromboplastic suspensions of various ages (multiples of fourteen days), prepared according to Quick's and Magath's techniques, at biweekly intervals and all from one progressively aging source of rabbit brain powder. This procedure also enabled us to ascertain the duration of the activities retained by Quick's and Magath's thromboplastin suspensions stored at 5° C., and to estimate the stability of suspensions freshly made from brain powders kept at this low temperature as compared with those stored at room temperature.

From the data presented in Table II, the two types of preparation thus tested exhibit decided variation in thromboplastic potency. It is apparent that the rabbit brain powder as prepared by Quick's technique of acetone dehydration is not only more potent in a fresh state than it is when prepared by the Magath incubator-desiccation method, but the saline suspensions made from the Quick powder retain their activity for a much longer period than those made from the Magath preparation. The slight decrease in thromboplastic activity of Quick's brain powder, as evidenced by the negligible prolongation of the prothrombin clotting time, is of no practical importance. It is, however, also evident that Magath's brain powder retains its thromboplastic potency in the same corresponding degree as Quick's preparation, since the prothrombin time obtained by a suspension made from aged powder is practically identical with that obtained by a similar solution prepared from fresh powder. In either instance, in order to safeguard retention of its potency, rabbit brain powder must be stored at icebox temperature in a well-stoppered vessel, since at room temperature changes detrimental to its activity invariably occur.

#### SUMMARY

Experimental data obtained in 14 series of observations made at biweekly intervals are presented, showing the relative potency and stability of thromboplastins made from both fresh and aging rabbit brain powders prepared according to the techniques of Quick and of Magath.

The results of our experiments demonstrate clearly the greater thromboplastic activity of rabbit brain powder prepared by the acetone-dehydration method of Quick than that of the incubator-dried preparation of Magath. Providing the preparation is stored at 5° C. in a tightly stoppered bottle, this superior potency of Quick's brain powder is retained for a period of at least six months. Likewise, if kept at 5° C., the suspension of rabbit brain powder, whether freshly made or as old as six months, is still capable of yielding pro-

thrombin clotting times practically identical with those obtained by it in its fresh state. The stability of both the dried rabbit brain powder, and its thromboplastic suspensions, which has been demonstrated in the present study, should dispel any doubt in regard to this particular feature and thereby encourage performance of prothrombin tests.

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## A NEW STABILIZING AGENT FOR NESSLERIZED SOLUTIONS\*

## ITS APPLICATION TO THE DETERMINATION OF UREA NITROGEN IN BIOLOGICAL FLUIDS

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IN THE visual comparison of nesslerized solutions, the eye of the observer is estimating extinction in a region of the spectrum to which the eye is least sensitive (absorption in the violet). For this reason, various substances such as gum arabic and gum ghatti can be added to stabilize the nesslerized solution without altering appreciably the precision of the visual comparison. The same substances, however, affect the photoelectric transmission value markedly. This is presumably the reason why some investigators in applying photoelectric techniques to the nesslerization of ammonia formed from blood urea have eliminated the gum stabilizing agent, but in doing this they have eliminated an important advantage enjoyed in the visual comparison, namely, stability of the transmission value of the nesslerized solution. Thus, Hoffman,<sup>4</sup> in eliminating gum ghatti, found it necessary to read his samples two minutes after color development and even then could read to only 40 mg. per 100 c.c. (0.016 mg. of urea nitrogen in 10 ml. of solution) of urea nitrogen in blood presumably because of rapid clouding of the sample. In addition, the transmission value of his blank is rather large, i.e., about 71 per cent. Nevertheless, the method is one of the most recent and successful in applying the photoelectric colorimeter to a direct nesslerization procedure for blood urea. In an effort to get back to the advantages of the visual comparison of nesslerized solutions and combine them with

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the advantages of the photoelectric colorimeter, we have sought a more efficient stabilizing agent. This paper sets forth what we believe to be our successful findings.

#### APPARATUS AND REAGENTS

*Photoelectric Colorimeter.* An instrument similar in design to the Evelyn colorimeter<sup>1</sup> was constructed with a sleeve of variable aperture to take a standard (16 by 150 mm.) pyrex tube rather than the large 25 ml. capacity tube used by Evelyn. However, any photoelectric colorimeter now on the market may be used instead of the instrument described.

*Colorimeter Tubes.* Tubes are selected from a large batch of pyrex (16 by 150 mm.) tubes and have graduation marks at 5 and 10 ml. They are chosen so as not to differ in transmission by more than 0.2 per cent.

*Filter.* The proper filter for the yellow nesslerized solution was selected from spectrophotometric data obtained with a Coleman-Bell double monochromator spectrophotometer. Using Corning glass filters, it can be composed by using 2 mm. of glass No. 585 with 3 mm. of glass No. 428. This combination gives a transmission band whose midpoint falls at about 440 m $\mu$ .

*Water.* Triple distilled, ammonia-free water was used for all reagents, standard solutions and dilutions.

*Liquoid* (Sodium polyanetholsulfonate). Hoffmann-La Roche product, 2 per cent solution in water.

*Urease.*<sup>6</sup> Treat 10 Gm. of permittit in a liter flask with 100 ml. of 2 per cent acetic acid. Decant off the acid, and wash the remaining permittit three times with distilled water. Add 150 ml. of 0.001 N sulfuric acid and 50 Gm. of jack bean meal and shake for fifteen minutes. Add 200 ml. of glycerol and shake in a shaking machine for one and a half hours. Allow mixture to settle for about one-half hour in the icebox. Then remove supernatant fluid and centrifuge it at 1,500 r.p.m. Transfer the supernatant fluid from the centrifuge tubes to glass stoppered bottles and place them in the icebox. This concentrated extract of urease will keep indefinitely.

*Blood Filtrate Reagents.* Tungstic Acid: This is a slightly more concentrated form of the Van Slyke and Hawkins<sup>9</sup> modification of the original Folin-Wu reagents and is made from two solutions: Solution A—Dissolve 111 Gm. of sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ ) in water and dilute to one liter. Solution B—Add 6 ml. of concentrated reagent grade sulfuric acid to 1,500 ml. of water and dilute the two liters.

For use, mix one volume of solution A with eight volumes of solution B. For blood filtrate, mix nine volumes of this mixture with one volume of blood.

Somogyi<sup>7</sup> reagents for zinc filtrate of blood: Solution A—10 per cent zinc sulfate: 100 Gm.  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  dissolved in one liter of solution. Solution B—0.5N sodium hydroxide. Ten milliliters of solution A should require between 10.8 and 11.2 ml. of solution B to produce a permanent phenolphthalein end point. To prepare 1:10 blood filtrate, take one volume of blood with seven volumes of water. Add one volume of solution A, mix, and then add one volume of solution B. Mix again and filter.

*Nessler's Reagent.* Sixty-eight grams of the dry Nessler salt (obtained from the Technicon Co., New York City) is dissolved in one liter of 5 per cent sodium hydroxide. Allow to stand several days in a pyrex flask in a dark closet before using. Use the supernatant fluid without disturbing the precipitate.

Since the intensity of color development varies with alkalinity of reagent, an optimum alkali concentration was selected at 5 per cent with the help of the data presented graphically in Fig. 1.

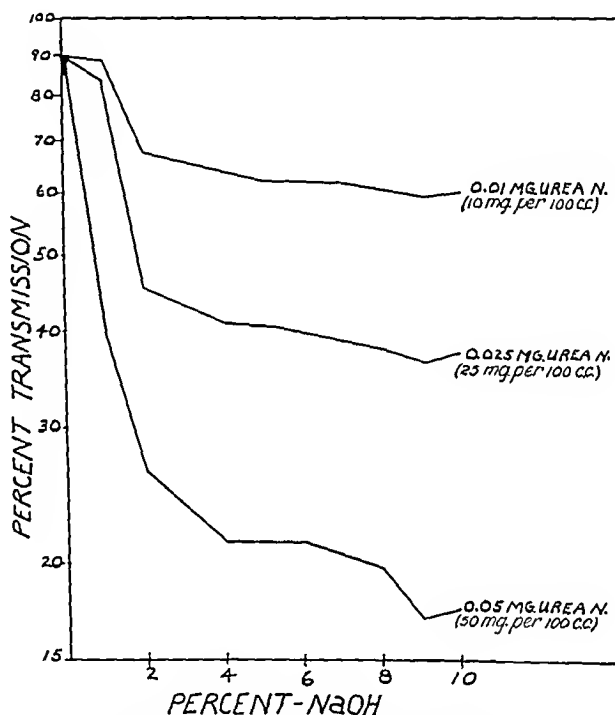


Fig. 1.—Alkali concentration of Nessler's reagent as per cent of sodium hydroxide solution (0.5 ml. Nessler's reagent in 10 ml. dilution).

*Standards.* Into volumetric flasks weigh out 3.821 Gm. and 2.145 Gm., respectively, of ammonium chloride and urea, dissolve in water, and dilute each to one liter. These were the stock standard solutions of which 1 ml. was equivalent to 1 mg. of nitrogen. (This value for the urea nitrogen stock standard was independently checked by a digestion, distillation, and titration procedure.) Appropriate quantities of 1:10 and 1:100 dilutions of these stock standards were pipetted out for the standards used in making the standard curves. The ammonium chloride and urea were recrystallized from reagent grade chemicals and dried at  $110^{\circ}\text{C}$ .

*Stabilizing Agent.* Since in nesslerized solutions clouding appears to be a process of gradual agglutination, the possibility of preventing it by means of blood anticoagulating agents was investigated. Liquoid (sodium polyanethol-sulfonate) proved to be particularly effective. It gives very low blanks, is obtainable in pure form, and requires only one drop of a 2 per cent solution to stabilize a 10 ml. volume of nesslerized solution. An idea of liquoid's ability to prevent

cracking of a nesslerized sample can be gained from the fact that 0.25 mg. of ammonia nitrogen in 10 ml. of nesslerized solution could be stabilized for a maximum of sixty minutes with one drop of 2 per cent liqoid, whereas the same solution without liqoid cracked within thirty minutes. In addition, the transmission value stabilized with liqoid falls much more slowly than with the more frequently used stabilizing agents, such as gum ghatti, gum arabic, gum senegal,<sup>2</sup> etc.

#### PREPARATION OF STANDARD CURVES

Our particular field of application of nesslerization is the direct determination of urea nitrogen in blood filtrate. Therefore, in the preparation of the standard curves an effort was made to have the standard solution conform to the actual condition of a blood filtrate. To this end, the average concentration of the tungstic acid remaining in a blood filtrate was determined<sup>3</sup> and found to be about one-fourth that used in the precipitation of the blood proteins. This quantity, therefore, was added to each standard solution. In Somogyi filtrates the concentration of the remaining zinc ion is small and can be approximated by adding 1 ml. of filtrate obtained from the mixing of the Somogyi reagents with distilled water used in place of blood.

*Standard Curves for Zinc Filtrates.*—Into tubes graduated at 10 ml., quantities of standard urea solution were pipetted so that the nitrogen concentrations (after treatment with urease) would be those contained in 0.1 ml. samples of blood, ranging from 5 to 70 mg. per 100 c.c. of urea nitrogen. One drop of urease extract was added to each tube and after fifteen minutes' standing at room temperature, the tube contents were treated with the Somogyi filtrate reagents, mixed, and filtered through Whatman No. 2 (9 cm.) filter paper. One milliliter of this filtrate was placed in a colorimeter tube and one drop of liqoid was added. Five milliliters of water and 0.5 ml. of Nessler's solution were next added, and the tube contents were diluted to 10 ml. and mixed. Each standard was run in triplicate and read in the colorimeter at various time intervals against a distilled water blank.

To gain an idea of the thoroughness of conversion of the urea to ammonia by this technique, quantities of ammonium chloride standards were pipetted into a second series of colorimeter tubes so that the ammonia nitrogen would be that contained in 1 ml. samples of blood filtrate (equivalent to 0.1 ml. blood) resulting from bloods ranging from 5 to 70 mg. per cent in urea nitrogen. One milliliter of zinc hydroxide filtrate obtained from the mixing of the Somogyi reagents (water replacing blood) was added to each tube followed by the addition of one drop of liqoid solution, 5 ml. of water and 0.5 ml. of Nessler's solution. The tube contents were diluted to 10 ml., mixed, and read in the photoelectric colorimeter at various time intervals. Each standard was run in triplicate. The transmission values of *both* these sets of standards fall on the same curve. The thirty-minute values are plotted in Fig. 2, where the coincidence of both sets of values is illustrated.

Another set of values was obtained for the same series of ammonium chloride standards used above, with 1 ml. of a 1:4 dilution of tungstic acid



filtrate reagent (water replacing blood) substituted for the zinc hydroxide filtrate used. The thirty-minute transmission values for these are also plotted in Fig. 2, and it is seen that they fall on a different curve. No urea nitrogen values are given on actual tungstic acid filtrates because the products of the enzymatic digestion of urea with the urease extract do not give a clear filtrate in the absence of blood or other protein-containing material. However, by analogy from the coincidence of the two standard curves based on standards containing zinc hydroxide filtrate and good recovery values of urea nitrogen using the tungstic acid curve, it is reasonable to assume that the curve based on ammonium chloride standards and tungstic acid will give transmission values very close to those that would be obtained from equivalent quantities of urea treated with urease.

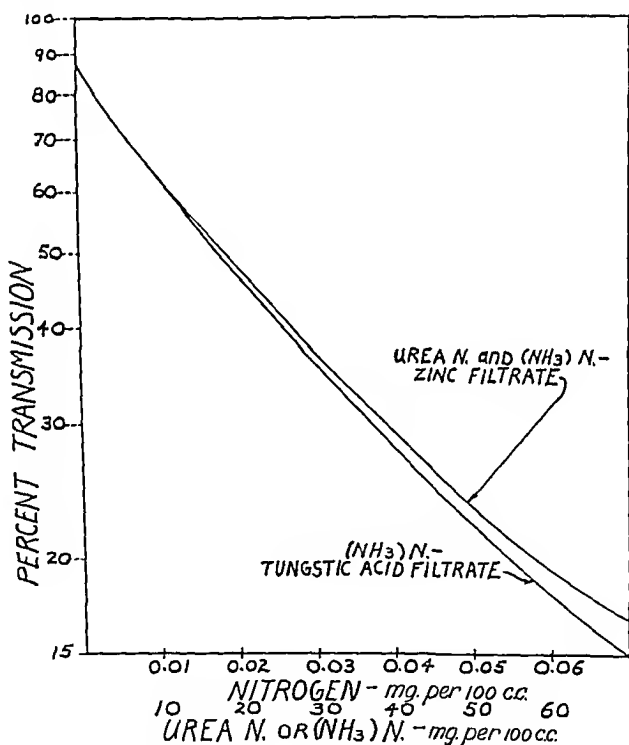


Fig. 2.—Transmission curves for colorimetric urea method for tungstic acid and zinc filtrates.

#### PROCEDURE FOR UNKNOWN AND RECOVERIES

In the routine estimation of blood urea nitrogen 1 ml. of blood is treated in a test tube with 1 drop of urease extract and left to stand at room temperature for fifteen minutes. Nine milliliters of tungstic acid reagent (if zinc filtrate is preferred, zinc filtrate reagents may of course be used) are then added, and the mixture is well shaken. After another five minutes' standing, filter through a Whatman No. 2 (9 cm.) filter paper. One milliliter of the filtrate is placed in a colorimeter tube, followed by 1 drop of liquor, 5 ml. of water, and 0.5 ml. of Nessler's reagent. The tube contents are then diluted to 10 ml.,

TABLE 1\*

DETERMINATION OF UREA NITROGEN IN BIOLOGICAL FLUIDS  
(0.01 mg. urea nitrogen is equivalent to 10 mg. per 100 c.c. urea nitrogen)

| SPECIMEN      | UREA NITROGEN IN SAMPLE<br>(MG. PER 100 C.C.) |                     | UREA NITROGEN ADDED<br>(MG. PER 100 C.C.) | TOTAL FOUND<br>(MG. PER 100 C.C.) | RECOVERED<br>(MG. PER 100 C.C.) | PER CENT RECOVERED |
|---------------|---|---------------------|---|-----------------------------------|---------------------------------|--------------------|
|               | DISTILLA-<br>TION                             | NESSLER-<br>IZATION |   |                                   |                                 |                    |
| Serum 1       |   | 27.4                | 10  | 38.0                              | 10.6                            | 106                |
|               |   |                     | 25  | 51.1                              | 23.7                            | 95                 |
| Serum 2       | 7.4   | 7.6                 | 5   | 12.7                              | 5                               | 100                |
|               |   |                     | 10  | 17.7                              | 10                              | 100                |
|               |   |                     | 25  | 32.7                              | 25                              | 100                |
| Serum 3       | 7.1   | 7.8                 | 5   | 12.5                              | 4.7                             | 94                 |
|               |   |                     | 10  | 17.5                              | 9.7                             | 97                 |
|               |   |                     | 25  | 32.4                              | 24.6                            | 98.4               |
| Blood 1       | 6.3   | 6.8                 | 5   | 11.7                              | 8.9                             | 98                 |
|               |   |                     | 10  | 17                                | 10.2                            | 102                |
|               |   |                     | 25  | 33.8                              | 27.0                            | 108                |
| Blood 2       | 12.5  | 12.5                | 10  | 24.1                              | 11.6                            | 116                |
|               |   |                     | 25  | 38                                | 25.5                            | 102                |
|               |   |                     | 50  | 61                                | 48.5                            | 97                 |
| Plasma        | 5.6   | 5.3                 | 5   | 10                                | 4.7                             | 94                 |
|               |   |                     | 10  | 15                                | 9.7                             | 97                 |
|               |   |                     | 25  | 30.7                              | 25.4                            | 101.6              |
| Cbst 1        |   | 7.6                 | 10  | 18.3                              | 10.7                            | 107                |
|               |   |                     | 25  | 33.8                              | 26.2                            | 104.8              |
| Cbs 2         | 12.5  | 13.4                | 5   | 19                                | 5.6                             | 112                |
|               |   |                     | 10  | 24.2                              | 10.8                            | 108                |
|               |   |                     | 25  | 38.8                              | 25.4                            | 101.6              |
| Cbs 3         | 7.2   | 8.1                 | 5   | 13.9                              | 5.8                             | 116                |
|               |   |                     | 10  | 18.5                              | 10.4                            | 104                |
|               |   |                     | 25  | 33.2                              | 25.1                            | 100.4              |
| Pleural fluid | 8.7   | 8.8                 | 5   | 13.6                              | 4.8                             | 96                 |
|               |   |                     | 10  | 18.3                              | 9.5                             | 95                 |
|               |   |                     | 25  | 34.7                              | 25.9                            | 103.6              |
| Urine 1       | 196.7   | 205                 | 100                                       | 306                               | 101                             | 101                |
| Urine 2       | 256.8   | 262                 | 50  | 313                               | 51                              | 102                |

\*Values given in this table are the average of three or more determinations.

†Cerebrospinal fluid.

mixed, and read in the colorimeter at the end of thirty minutes, or at whatever time interval the standard curve is made. Other biological fluids are treated in the same way. However, one to ten dilutions are first made on urine specimens to bring their urea nitrogen content down somewhere within the range of the standard curve. The diluted sample is then treated exactly as for blood. Occasionally samples will be obtained with urea values so high that it will be desirable to repeat the determination on a smaller sample. If, however, a smaller sample is not available, an aliquot ( $\alpha$ ) of the 10 ml. volume in the colorimeter tube is taken and placed in another colorimeter tube, and 0.5  $(1 - \frac{\alpha}{10})$  ml. of Nessler's reagent is added. The tube contents are then diluted to 10 ml., mixed, and read in the colorimeter. If the extra Nessler's reagent is omitted, the trans-

mission value of the diluted sample will be too high. By this means the range of the method can be extended to ten times the range of the standard curve, i.e., 700 mg. per 100 c.c.

In Table I are given the urea nitrogen values obtained from tungstic acid filtrates of various kinds of biological fluids and urea nitrogen recoveries on these same fluids. The base values of the urea nitrogen content of these various samples are compared with those obtained by distillation and titration of the ammonia resulting from the urease treatment of the same filtrates. Values obtained by both methods are seen to be in good agreement. The recoveries, together with these compared values, are good enough to justify the use of the tungstic acid-ammonium chloride standard curve.

#### DISCUSSION

The extra buffer solution that is customarily employed in urea determinations to provide the optimum pH for action of the enzyme, urease, is omitted, because with the concentrated stable extract of urease used, it adds nothing to the completeness of conversion of the urea present.

Tungstic acid filtrates instead of zinc filtrates are prepared in this laboratory where daily filtrates sometimes number in excess of 100, because they require only a single reagent, whereas zinc filtrates require at least two, and with the original Somogyi technique, three, if the distilled water for the proper volume dilution is considered. In using tungstic acid filtrate we did not encounter the high blanks of which Hoffman<sup>4</sup> complained.

Since the transmission value of a nesslerized solution falls slowly, the standard curve should be made for a definite time interval, and the unknowns should be read at this interval.

In the practice of photoelectric colorimetry some writers<sup>8</sup> criticize the use of standard curves because they feel that the conditions under which the curve is prepared at one time, and those under which an unknown is read at another, are never exactly duplicated, and, therefore, choose to compare their unknown with a freshly prepared standard, and to get their result from the familiar inverse proportion based on the Bouger-Lambert-Beer law. This is sound practice when the law holds. In defense of our using a standard curve, we wish to point out that in many cases the Bouger-Lambert-Beer law holds only in a limited range of lower concentrations and deviates considerably at higher concentrations. Therefore, calculations based upon it often contain more error than would be introduced by use of a standard curve whose duplicability had been checked over a long period of time. The standard curves illustrated in this paper have been used and checked over a period of two years and furnish, we believe, an example of a set of conditions that can be sufficiently duplicated to validate their use.

Although the standard tungstic acid curve presented is especially prepared for blood filtrates, and an amount of tungstic acid is placed in the standards that approximates that in blood filtrates, the curve is usable for estimating the urea in tungstic acid filtrates on biological materials other than blood. This is apparent from the results given in Table I, which were obtained by use of this curve.

## SUMMARY

A new stabilizing agent, liquid, is offered for nesslerized solutions that gives very low blanks and stable transmission values. The technique is applied to urea determinations on the filtrates of biological fluids that have been treated with urease.

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# MEDICAL ILLUSTRATION

## FLUOROGRAPHY\*

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### DEFINITION

FLUOROGRAPHY is the process of recording, by use of an optical system and light-sensitive film, the image formed upon a fluorescent screen (1) by a differentially absorbed beam of radiant energy, usually roentgen rays; or (2) by a stream of directed electrically charged particles. The process, then, includes the photographic recording of fluoroscopic images found in medical or industrial radiology, the photography of television images, the photography of cathode-ray oscillograph patterns, and similar images. Fluorography definitely does not encompass all types of photographic recording in which fluorescence is utilized. For example, contact radiography in which a light-sensitive film is most frequently, but not necessarily, sandwiched between two fluorescent screens is not considered to be fluorography; nor are such methods as described by Dershem,<sup>1</sup> wherein a directed beam of fluorescent x-radiation is used to record the distribution of certain elements in a thinly sectioned specimen, by selecting as the fluorescing substance an element whose characteristic K-radiation corresponds closely to the K absorption limit of the element to be detected; nor, in spite of the similarity of name, is the so-called fluoroscopic process,<sup>2</sup> invented by W. S. Marx, Jr., in 1938 in any way related to fluorography. This latter process is a special procedure for producing so-called "high-light" half-tone screen negatives for use in photomechanical reproduction.

The process now called fluorography is, however, known also as indirect radiography, fluoroscopic screen photography, fluorophotography, miniature radiography, fluororadiography, microradiography, and roentgen photography, and at one time was called photofluoroscopy. It is interesting to note that the term which has been adopted through usage is the most simple. At a time when there was some question as to the etymologic correctness of the term fluorography, I approached the heads of the English departments in three leading United States universities with the question independently, and in each case, after learning the nature of the process and reviewing the names which had at one time or another been used to describe it, these authorities selected fluorography as the most logical term to adopt.

### HISTORY

Roentgen discovered x-rays in 1895. In the following year several means were devised for photographing the fluoroscopic image excited by these rays.

\*From the Agfa Ansco Product Information Department, Binghamton.  
Read at the Meeting of the Biological Photographic Association, September, 1941.

MacKay<sup>3</sup> described one method in March, 1896; Bleyer<sup>4</sup> constructed his photo-fluoroscope in April, 1896; Battelli and Garbasso<sup>5</sup> also described a method in January of the same year. In 1897 Porcher<sup>6</sup> published an article entitled, *Photographie de l'image fluoroscopique*. In 1897 McIntyre<sup>7</sup> published an article describing indirect radiographic motion pictures which he accomplished during a series of experiments in 1896 by photographing the shadow image formed on a potassium platino-cyanide fluorescent screen. At this period there was a sharp turning point with respect to the manner of making radiographic records. Initially, the photography of the shadow image formed on the fluorescent screen was the preferred method, but Porcher<sup>6</sup> and others were of the opinion that the results obtained in indirect radiography were unsatisfactory, and hence the direct method gained favor. A few years later Köhler<sup>8</sup> anticipated the promising future of fluorescent image photography; in 1909 Biesalski and Köhler<sup>9</sup> outlined a practical method for conducting the work in which were utilized a calcium tungstate screen (blue fluorescence) an F/2 lens, and a mirror permitting the camera to be placed outside the direct beam of roentgen rays. This revival of the indirect procedure by Köhler gained momentum as time went on, and within a few years nearly all the problems involved in the process had been recognized.

Caldwell<sup>10</sup> in 1911 published an important paper on indirect radiographic still photography and cinematography in which he set down practically all the essential principles concerning the application of fluorography. He employed a 4 by 5 inch camera and a F/4.5 Cooke lens in conjunction with a "Gehler Folie" for still work. The screen that Caldwell used had a short phosphorescent afterglow which, as pointed out by Hirsch,<sup>11</sup> anticipated the most suitable screen available today for fluorography—the "fluorazure" screen, more properly known as the photo-roentgen screen, which has an afterglow also, but as used in practice it does not persist long enough to interfere with the clarity of the recorded image.

Loman and Camandon<sup>12</sup> were the first to utilize an extremely wide aperture lens in fluorography. In 1913 they employed a lens of F/1.55 relative aperture for motion pictures of the fluoroscopic image.

It is doubtful if much of this early photography of the fluoroscopic image was successful, especially the motion picture photography.\* The problems involved were well understood, but the limitations were too severe to permit satisfactory results. It was not until lenses of wide aperture were generally available, until photographic emulsions were made faster, and until more efficient roentgen-ray tubes and fluorescent screens were devised that fluorography produced results worthy of evaluation.

#### APPLICATIONS

Although categorically fluorography includes the photographing of fluorescent screen images of any type, the widest field of application is found in medical radiology. Nearly the entire development of the process has been cou-

\*This view is based somewhat upon the private communication from S. L. Warren, Rochester, N. Y., reported in the *Science of Radiology*, page 204, edited by Otto Glasser (1933), in which it is claimed that after five years' labor it had been found impossible to achieve a satisfactorily sharp image in cinefluorography with the use of lenses of very wide aperture. This statement had reference to the publication of Luboschew,<sup>25</sup> who had reported good results in 1929 by means of a lens of F/0.65 relative aperture.

tributed by medical researchers and roentgen-ray technicians, their reports being published in medical and allied journals. In medical radiology there are two distinct applications of fluorography, single and serial exposures. Both applications are important, and the same technical problems in attaining suitable results are involved. In serial fluorography the successive exposures frequently can be made sufficiently rapid to permit the taking and subsequent projecting of roentgen-ray motion pictures.\* This branch is called cinefluorography.

Single exposures of the fluorescent image are made for purposes of record or diagnosis, or both. For group studies of large populations, as in thoracic surveys, fluorography represents a distinct advantage over contact radiography, especially from an economy standpoint. This branch is known as collective fluorography and the individual records are called fluorograms.

In Germany the application of fluorography in medical diagnosis is regarded as limited to the determination of lung shadows, disease centers of sizeable area, and the frequent detection of active tuberculosis. It is not considered suitable for showing satisfactory bone structure, nor early infiltrations in pulmonary disease, such as early tuberculosis and early silicosis.<sup>13</sup> However, Hirsch<sup>14</sup> in this country, with reservation similar to that of the Germans but with a somewhat more optimistic outlook, states that fluorography is: 1. Applicable to the study of the skeleton in delineating *gross* bone changes and deformities. 2. Useful in gastrointestinal surveys, particularly in the study of the colon and in motility tests. 3. Suitable for showing clearly the position and shape of the heart and, upon applying proper correction factors, the heart shadow diameters can be determined according to the usual method. 4. Valuable in the study of the lungs, particularly for tuberculous changes, the records being sufficiently clear to permit the differentiation of the normal from the abnormal lung.

Manoel de Abreu,<sup>14</sup> who in 1936 inaugurated the first installation of fluorographic apparatus for the purpose of carrying out a mass examination in Rio de Janeiro, also is of the opinion that fluorograms yield useful diagnostic evidence. His enthusiasm for the process is largely responsible for the installation of numerous fluorographic outfits throughout Brazil and other South American countries. Abreu's influence was not limited to South America, for he acquainted D. O. N. Lindberg of Decatur, Ill., with the success of his work, and Lindberg brought the method to the United States in 1937.

Cinefluorography has been found by Stewart and Ghiselin<sup>15</sup> to be safer and more effective than other radiographic procedures in the study of abnormal diaphragm and mediastinal movements in the understanding of pulmonary disease. In an earlier paper these same workers<sup>16</sup> reported that cinefluorography had several advantages over direct fluoroscopic examination in the study of many moving organs, such as the heart. They claimed, in addition to other factors, that the motion picture was even more defined than was observation with the best possible fluoroscopic screen. In a similar vein Böhme<sup>16a</sup> expressed the merits of cinefluorography in studies of the heart. He used the process to

\*So-called x-ray or roentgen-ray motion pictures are not entirely "real." The motion picture record is actually only a repetition of a single eye which gives the effect of a continuous action. However, single cycles of an organ do not constitute a full study of the organ, since some eyes may be normal whereas others may be pathologic.

demonstrate his discovery regarding the important function of the auriculo-ventricular diaphragm in the propulsion of blood. He was able to make as high as 150 exposures per second for short intervals of time, three to four seconds.

Outside the medical fields fluorography has had a scattered but significant application. With the coming of an increased use of fluoroscopic roentgen-ray examinations in industry for quality control, it is natural to assume that there would be a corresponding increase in the number of photographic records desired. Moreover, the growth in the uses for cathode-ray tubes has occasioned an increase of interest in retaining records of traces in certain experimental and even in routine studies.<sup>17</sup> Dudley<sup>18</sup> has published an article on the photographic recording of television images. Undoubtedly interest in this branch of fluorography will gain in application as television becomes more practical.

There are many optical and photographic problems which pertain to fluorography, and in view of the likelihood that the process will find wider application in medicine, industry, and science, it is deserving of more extensive study.

#### FACTORS IN OBTAINING OPTIMUM RESULTS

The aim in fluorography is specifically to reproduce as accurately as possible the fluorescent screen image with a maximum of sharpness and with adequate contrast to permit evaluation of details. In medical applications an additional aim, of course, is to replace radioscopy examination by a less dangerous method and, in addition, to offer better documentation.<sup>19-21</sup> Whether or not the aims are satisfied is determined by the conditions of operation and the acumen with which the situation as presented by the limitations of equipment is met. First results are often disappointing, simply from a lack of appreciation for the problems involved. The alacrity with which an individual technique is evolved will be influenced vastly by the extent of one's knowledge of the overall problem.

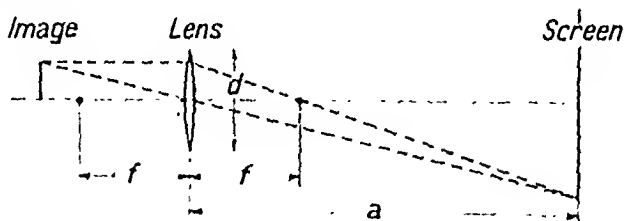
There are several factors that govern the quality of the result.<sup>13, 19, 22-24</sup>

1. The efficacy of the radiant energy or electron stream to excite fluorescence. In the case of medical radiology, the energy of the incident roentgen-ray beam is limited by the capacity of the tube and by the tolerance of the patient's skin.
2. The light-producing power of the fluorescent screen, which varies qualitatively and quantitatively according to:
  - (a) The chemical composition of the screen.
  - (b) The color energy distribution of the fluorescent radiation.
3. Sharpness factors which are dependent upon additional screen characteristics, namely,
  - (a) Graininess of screen structure, whether coarse or fine.
  - (b) Time lag of screen.
4. Characteristics of the camera lens which include:
  - (a) Light-gathering power; that is, the lens speed.
  - (b) Sharpness of the lens at wide apertures.
5. Sensitivity of the film to light energy emitted by the screen.

It is not convenient to discuss these various factors in the order given. Because the process of fluorography involves the use of an optical system, it is best



to outline first the limitations which this imposes. Moreover, it is the use of an optical system that chiefly distinguishes fluorography from normal roentgenographic procedures in which the recording film and screens are in actual contact. However, it is the matching of the quality of the large roentgenogram, made in the normal manner, by the indirectly made reduced image that more or less establishes the quality criterion for fluorography.



$d$  equals aperture of lens  
 $f$  equals focal distance  
 $a$  equals screen-lens distance

$$\frac{\pi d^2}{4} : 2\pi a^2 \quad \text{or} \quad \frac{d^2}{8a^2}$$

Fig. 1.

In contact radiography\* the entire radiation within an angle of 180 degrees, and even more when screens are used on both sides of the film, is utilized in exposing the film. With a lens system the amount of radiation received by the film is limited at the outset by the acceptance angle of the objective. This requires, of course, a brighter screen image in fluorography than is necessary in normal roentgenography, but since this in turn demands an increase in the total energy output of the roentgen-ray tube, it is desirable to employ the fastest possible photographic objectives. Lenses of relative aperture in the range of from  $F/0.53$  to  $F/0.625$  have been used in cinefluorography,<sup>25-27</sup> but such lenses are not readily available nor could they be made of sufficient focal length to cover a large field. The Zeiss Biotar objective is available with a maximum  $F$  value of 0.85, 4.5, or 5.5 cm. focal length, but the overall sharpness cannot be regarded as entirely satisfactory. Much more satisfactory results are obtainable with the Zeiss Biotar  $F/1.4$ , 7 cm. focal length, or with the standard Contax, Leica, or Ektar  $F/1.5$  to  $F/2.0$  objectives. Furthermore, it is preferable to have even these objectives surface-treated to minimize internal light reflections, otherwise the resultant loss in image contrast would have to be gained through an increase in the brilliance of the screen image or by the use of a film of exceptionally steep exposure-density gradient.

A mathematical approach to the determination of the conditions for optimum results has been made by Bonwers,<sup>10</sup> which is an expansion of a former work concerning the so-called law of uniformity.<sup>28</sup> This law is concerned with the factors which are causes for unsharpness in the normal roentgenogram of objects in motion and specifically states that optimum conditions are reached when the factors are equal; thus,

$$U_m = U_g = U_n \quad (1)$$

\*It should be noted that television and other cathode-ray tube tracings can be recorded by contact methods but less conveniently than by means of a camera.

where  $U_m$  is the unsharpness resulting from movement,  $U_g$  is the geometrical unsharpness, and  $U_s$  the unsharpness caused by screen factors. Mean values of  $U_g$  and  $U_m$  are assumed since these factors are not constant for the whole image. The law of uniformity is based upon the fact that the product of  $U_m$ ,  $U_s$ , and  $U_g$  is a measure of the amount of light reaching the film and, for a given value of the product, a given image density is achieved. However, the sum of three numbers with constant product is smallest when the numbers are equal; therefore, we get equation 1, since the total unsharpness is the sum of the three unsharpness factors.

Although the law of uniformity originally applied to normal radiography, it is equally applicable to fluorography, provided, as in the case of normal radiography, that a rotating anode tube is used. For example, if we assume that proper radiographic conditions for optimum results are established, we can eliminate one contact fluorescent screen and photograph the image on the remaining screen with a lens and camera. With the fastest practical lenses the light per square centimeter incident upon the film in the camera is only one-tenth that per square centimeter incident upon the film when in contact with two fluorescent screens. Accordingly, if a film of the same sensitivity as the normal roentgen-ray film were used, and the energy output from the tube remained constant, then an exposure increase of twenty times would be required for the camera exposure. However, such a vast increase in time of exposure would increase  $U_m$  to an intolerable extent and at the same time the life of the roentgen-ray tube would become endangered, not to mention the danger involved by giving the patient such a large roentgen-ray dose.

Fortunately, the law of uniformity can be applied to show what changes are required to establish exposure conditions for optimum results. Since

$$U_m = U_g = U_s$$

and since in the case at hand

$$U_m \times U_g \times U_s = 20X$$

the new conditions will be best if each unsharpness factor is multiplied by

$$\sqrt[3]{20} = 2.7$$

in order to increase its product by a factor of twenty. These conditions could be met by the use of a tube of wider focus, by means of a somewhat coarser grained screen of greater layer thickness, and by increasing the time of exposure now by only 2.7 times. The unsharpness in the final reduced image is then only 2.7 times greater than in the direct double screen image. From this it is apparent that the loss of sharpness in the small image is determined by the ratio of the amount of light per square centimeter in the small image,  $I'$ , to the amount of light,  $I$ , that would be available per square centimeter of film surface in the direct method. Therefore, if we denote the increase of unsharpness by the factor  $k$ , its magnitude is determined by

$$k = \sqrt[3]{\frac{I}{I'}} \quad (2)$$

As previously mentioned, this deduction is based upon equal film sensitivities which at present, at least, can be assumed to be approximately true. There are

also additional factors that are ignored, otherwise the treatment becomes unduly complicated. For example, no regard is given the fact that fluorograms are projected and accordingly can be of a lower density level than normal roentgenographs.

The calculation of the ratio of  $I' : I$  can now be considered. In Fig. 1 the geometric relation between the screen, camera lens, and reduced image plane are shown. If  $d$  is the aperture of the lens,  $f$  its focal distance, and  $a$  the screen lens distance, then the relation of the light passing through the lens to the total light emitted by the screen is

$$\frac{\pi d^2}{4} : 2\pi a^2 = \frac{d^2}{8a^2}$$

If the reduction is  $n$  times, then the surface area over which the light passing through the lens is distributed will be  $\frac{1}{n^2}$  that of the screen; consequently, the brightness of the reduced image becomes  $\frac{n^2 d^2}{8a^2}$  times that in a film in contact with one screen, or  $\frac{n^2 d^2}{16a^2}$  times that in a film between two screens. But,

$$n \cong \frac{a}{f}, \text{ so } \frac{I}{I'} = 16 \left( \frac{f}{d} \right)^2 \quad (3)$$

Now, by equation 2 we obtain for the increase in unsharpness the factor,

$$k = \sqrt[3]{16 \left( \frac{f}{d} \right)^2} \quad (4)$$

It so happens that it is an advantage to use a higher voltage for fluorography than is favorable for direct roentgenography. By so doing an increase in sharpness follows, since the tube output is increased and results in a smaller product for  $U_s \times U_m \times U_g$  for a given image density. This increase in tube voltage also decreases the factor  $I/I'$ . Now if in addition to this, the focus screen distance is reduced, a gain in light intensity can be effected. As a matter of fact, the sharpness can be doubled in the small image by increasing the tube voltage by 30 per cent and by reducing the focus-screen relation to one-half.

A new unsharpness factor can be established if these compromises are made, since equation 4 becomes

$$k' = \sqrt[3]{4 \left( \frac{f}{d} \right)^2} \quad (4a)$$

when the tube voltage is increased 30 per cent and the focus-screen distance is halved.

The values of  $\frac{f}{d}$ , as we have noted, are generally not smaller than about 1.5. Substituting this in formula 4a, the unsharpness factor becomes 2.08. This amount of unsharpness, according to Bouwers, corresponds to the relative sharpness acquired with rotating and stationary anode tubes.

From these calculations, which comply rather closely with practice, it is clear that there is room for improvement in the comparative quality of fluoro-

grams as made in medical roentgenography. However, the present results under the best conditions compare favorably with the quality of direct roentgenograms of an earlier period, and so for many applications can be considered adequate.

*Sharpness Factors Dependent Upon Screen Characteristics.*—In the foregoing calculations Bouwers<sup>28</sup> eliminated the unsharpness resulting possibly from the lens and film by assuming that these would be small in comparison to the unsharpness caused by other factors, even under conditions conducive to ideal reproduction. (The elimination of the lens as a factor becomes more acute in cinefluorography, for here the demand for wider aperture and greater sharpness are both encountered.) He assumed that miniature camera films of the fine grain type would be employed, but actually in modern fluorographic practice special films are coming to the fore. The films have an exceptionally high sensitivity to the fluorescence emitted by available photo-roentgen screens, and in addition are of rather steep inherent contrast. However, the graininess of these films compares favorably with the graininess of modern high speed roentgen films. Nevertheless, in relation to the unsharpness introduced by the grain structure of the screen as well as other factors, the film is well within the permissible limits as far as these concern the ability of the film to register fine detail, viz., resolving power. For example, if we assume the unsharpness in a normal radiographic image to be of the order of 1 mm.,\* the magnitude of the unsharpness in a 15 diameter reduction would be about 0.07 mm. The resolving power of the film, then, is required to be less than 10 lines per millimeter, which is far inferior to present emulsion standards even for coarse grain films. This means that the extent factor of unsharpness here rests with the nature of radiographic imagery and the fluorescent screen. The size of the focal spot and other factors determine the sharpness of the image primarily, but the coarser the grain of the screen the less fine detail will be discernible. However, since increased light emission accompanies an increase in grain size of the screen, there must be an optimum condition that can be met. Actually, the emission characteristics of the screen should be a maximum for a given amount of excitation, but this should not be carried to a point that will give an unsharpness greater than that presently accepted in normal radiography. It would be ideal, of course, to have a screen of high emissivity and of very fine grain structure.

The phosphorescent lag of the screens used in modern roentgen-ray practice is generally nil. And certainly there can be no appreciable lag in the screens adapted for cathode-ray tubes in television and in oscillograph manufacture. However, as previously pointed out, with the special blue fluorescing Patterson photo-roentgen screen there is a persistence of image, but this is of no consequence in the methods used in still fluorography. If anything, the afterglow slightly increases the image density without causing deleterious effects. Screens that are employed in cinefluorography can exhibit no persistence of image. For this work the Patterson fluoroscopic, type B, screen is excellent. A special study of the lag effect has been undertaken by Hirsch.<sup>29, 30</sup> He found that by permitting the camera shutter to remain open five minutes after the short ex-

\*It should not be inferred from this discussion that the screen is the chief cause of unsharpness in the normal radiographic image. The screen unsharpness is of the order of 0.15 to 0.17 mm., according to measurements made by the Patterson Screen Co. These data are corroborated by measurements published by H. Mermagen, x-ray technician, September, 1939.

citation period and by using a special screen with pronounced lag, an appreciable gain in image density resulted, but only at a sacrifice of overall contrast.

*The Light-Producing Power of the Fluorescent Screen.*—The exact chemical composition of commercial fluorescent screens is not available, nor is such information of particular value. It is known that certain compounds, when properly crystallized and dispersed, exhibit fluorescent properties to a greater or lesser extent, and that the color of the light varies according to the particular substance used. Roentgen-ray intensifying screens are dependent upon calcium tungstate for their fluorescence. Today these screens exhibit no lag, although at one time this represented a considerable problem. Minute quantities of certain metals are known to eliminate afterglow; for example, as small an amount as  $10^{-6}$  Gm. of nickel added to a zinc sulfide layer is sufficient. However, regular intensifying screens are not used in fluorography. Instead, zinc sulfide fluoroscopic screens of either blue-green or blue fluorescence are preferred. The color energy distribution of the fluorescence for the various types of screens is given in Table I.

TABLE I

| TYPE   | SPECTRAL EMISSION   |
|--|---|
| Intensifying screens - - - - -<br>(Calcium tungstate)                      | 3,800 to 5,200 Å.<br>(Maximum band at approximately 4,300 Å.)   |
| Fluoroscopic screens - - - - -<br>(Patterson photo-roentgen or fluorazure) | 3,900-4,900 Å.  |
| Fluoroscopic screens - - - - -<br>(Type B)                                 | 5,200-6,000 Å.<br>(No sharply defined maximum band is exhibited by either type of fluoroscopic screen.) |

*Sensitivity of Film to Light Energy Emitted by Screen.*—Either standard film products or special films can be used in fluorography. It is preferable to use a film of high contrast, and one which is especially sensitive to the radiation emitted by the fluorescent screen. A film of this type, developed by Agfa Anseo, is known as "Fluorapid."

The spectral sensitivity of Fluorapid film is represented in the wedge spectrogram, Fig. 2. It is readily apparent that the major part of the sensitivity of the film is between 4,000 and 5,700 Å. This range is suitable for both the photo-roentgen and type B fluorescent screens.

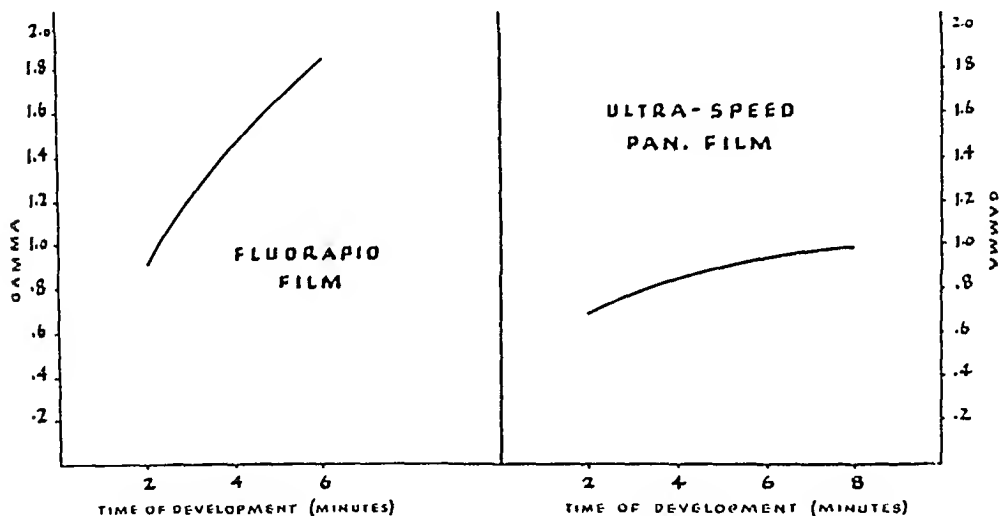
The total sensitivity of Fluorapid film to the light of the photo-roentgen screen is especially high, being equal to, or slightly faster, in the threshold region than in the high-speed roentgen-ray film emulsions. In addition to having a speed equal to the fastest emulsions available today, it also has a relatively steep exposure-density gradient. As shown in Table II, the film can be developed in roentgen-ray type developers to gamma values of over 1.8 which, in comparison to a regular miniature camera type film of similar speed, is exceptionally good.

Other types of films can be used in place of Fluorapid film when the Patterson type B fluoroscopic screen is used. Since the spectral emission of this

screen falls between 5,200 and 6,000 Å., high speed panchromatic emulsions, such as Agfa Ultra-speed, are satisfactory, as well as Fluorapid. For cine-fluorography, a reversible type film, such as Agfa Triple S Pan reversible, is suitable. Films of the Fluorapid type are, however, generally better than other films for both the photo-roentgen and the Patterson B screen and, in addition, have been found excellent for the photography of television images and cathode-tube tracings.\*

TABLE II

| TIME OF DEVELOPMENT IN AGFA NO. 30<br>DEVELOPER, 68 F° | GAMMA FOR TIME-SCALE EXPOSURES, 5,400 K. LIGHT<br>SOURCE, TANK DEVELOPMENT, AGITATION 5 SECONDS<br>PER MINUTE |                 |
|--|---|-----------------|
| Minutes  | Fluorapid   | Ultra-Speed Pan |
| 2  | 0.95  | 0.65            |
| 4  | 1.5   | 0.85            |
| 6  | 1.88  | 0.95            |
| 8  |   | 1.0             |



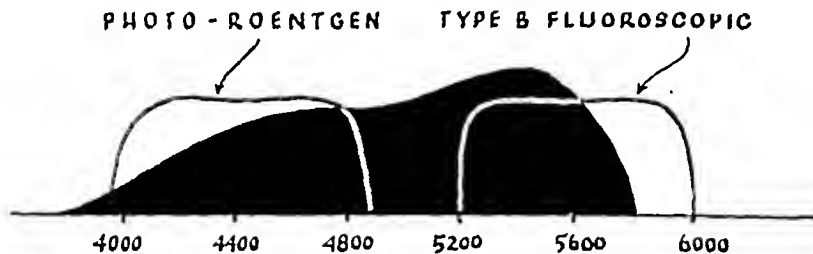
FILMS TANK DEVELOPED IN A-30 AT 68°F

## METHODS, APPARATUS, AND MATERIALS

The actual making of a fluorogram consists simply of photographing the image of the fluorescent screen with a Leica, Contax, or other similar camera, equipped with an F/1.5 or F/2 lens, and loaded with a suitable film. In radiographic practice the roentgen-ray tube, the patient, the fluorescent screen, and the camera are placed in direct line with each other and properly spaced. The most simple arrangement, of course, is to place the camera on a firm support and direct it toward the screen. It is advisable, if not absolutely necessary, to place a thick piece of lead glass in front of the camera lens to absorb any of the direct roentgen rays that might fog the film. As previously mentioned, to avoid the effect of the direct roentgen rays Biesalski and Köhler<sup>9</sup> photographed a mirror

\*By using a Contax camera, F/1.5 lens and Agfa Fluorapid film, cathode-ray oscillograph signals as short as 0.0003 second were recorded with good density. The size of the screen image on the film was about  $\frac{3}{16}$  inch. Previous to the actual exposure for recording the signal, an image of the dimly illuminated screen was produced on the film with a one second exposure.

image of the screen which permitted the placement of the camera outside the beam of direct radiation. However, this practice has not been followed by modern workers, such as de Abreu<sup>14</sup> and Hirsch,<sup>11, 20, 30</sup> who utilize a straight line system of almost identical specifications. The apparatus of de Abreu is mounted somewhat more elaborately than that of Hirsch, but the method of Hirsch is far more readily set up by the average clinical photographer. An illustration of his apparatus is shown in Fig. 3, A and B.



**WEDGE SPECTROGRAM (SOLID)  
OF FLUORAPID FILM  
(TUNGSTEN)**

Fig. 2.

A 35 mm. type camera\* is preferable for fluorography in medical roentgenography for the following reasons: 1. The new type films, such as Agfa Fluorapid, are available only in this size. 2. Lenses of F/1.5 or F/2 relative aperture are standard equipment with several 35 mm. cameras. 3. The fluorograms in survey studies are made in great numbers and rapidly. This means that a camera which can make sequence exposures easily and rapidly is required, as well as a conveniently developed film. 4. The fluorograms are viewed by projection and projectors readily available and convenient to use. 5. The small film sizes are economical.

The lens of the camera should be of the highest quality in order to give an acceptably sharp image over the entire 24 by 36 mm. film area at wide open apertures. Hirsch lists the available lenses of F/1.5 relative aperture, or slightly faster, that can be considered. It would seem advisable to test any lens under consideration by a procedure such as that outlined by Gardner<sup>31</sup> in the Bureau of Standards Research Paper No. C 428. This publication not only provides a simple and accurate lens testing procedure, but also includes a carefully prepared lens test target.

It is not the purpose of the present discussion to delve into the qualities of various roentgen-ray equipment for fluorographic work. The general assump-

\*Rather recently special 4 by 5 inch fluorographic equipment has been introduced for which ordinary high-speed roentgen-ray film has been found quite adequate. Such cameras are now employed by the army for group radiographic examinations, but as yet the 4 by 5 inch size has not won favor over the 35 mm. record for obvious reasons. Nevertheless, when lenses of high speed capable of covering a 4 by 5 inch field are generally available, this size is likely to surpass the 35 mm. method in popularity. For example, from a comparative study made by Plunkett, Weber, and Katz<sup>32</sup> it was concluded that 4 by 5 inch images were more suitable because of greater accuracy in reading; slightly better economy, resulting from fewer re-examinations; fewer 14 by 17 inch examinations to effect correct diagnosis; and less strain visually in examining 4 by 5 inch negatives than in the case of 24 by 36 mm. images.

TABLE III

| WORKER               | CAMERA AND LENS   | TYPE OF SCREEN  | DISTANCE                                   |                | PEAK (kv.) | mm.        | TIME (SECONDS)               | FILM                                 | REFERENCE            | VIEW                                      |
|----------------------|---|---|--|----------------|------------|------------|------------------------------|--------------------------------------|----------------------|---|
|                      |   |   | CAMERA TO SCREEN                           | TUBE TO SCREEN |            |            |                              |                                      |                      |   |
| Hirsch               | Zeiss Sonnar 35 mm. F/1.5   | Fluorazure or Patterson B   | 90 cm.                                     | 80 cm.         | 88         | 150        | 1/10 sec.                    | Agfa Fluorapid Eastman Special       | No. 29, p. 6         | Postero-anterior of chest                 |
| Hirsch               | Zeiss Sonnar 35 mm. F/1.5   | Fluorazure or Patterson B   | 90 cm.                                     | 70 cm.         | 96         | 80         | 4 sec.                       | Agfa Fluorapid Eastman Special       | No. 29, p. 7         | Gastrointestinal                          |
| Hirsch               | Zeiss Sonnar 35 mm. F/1.5   | Fluorazure or Patterson B   | 90 cm.                                     | 80 cm.         | 90         | 80         |                              | Agfa Fluorapid Eastman Special       | No. 29, p. 7         | Extremities                               |
| Hirsch               | Zeiss Sonnar 35 mm. F/1.5   | Fluorazure or Patterson B   | 90 cm.                                     | 60 cm.         | 90         | 160        |                              | Agfa Fluorapid Eastman Special       | No. 29, p. 7         | Skulls                                    |
| Agfa Ansco           | Leica 35 mm. F/2  | Fluorazure or Patterson B   | 90 cm.                                     | 102 cm.        | 80         | 350        | 1/20<br>1/15<br>1/10         | Agfa Fluorapid                       |                      | Thin chest<br>Normal chest<br>Thick chest |
| X-ray technician     |   |   |  |                |            |            |                              |                                      |                      |   |
| de Abreu             | F/1.5   | Zinc Sulfide  | 90 cm.                                     | 60 cm.         | 80         |            |                              | Agfa Isochrom                        | No. 21               | Chest                                     |
| Holfelder            | F/1.5   | Zinc Sulfide  | 90 cm.                                     | 70 cm.         | 80         |            |                              | Agfa Isochrom                        | No. 32               | Chest                                     |
| de Abreu             | Zeiss F/1.5   | Patterson G. E. Siemens Super-Astral                                  | 90 cm.                                     | 60 cm.         | 55<br>50   | 50<br>100  | 0.2-0.3<br>0.03-0.05         | Kodak Super XX<br>Agfa Isopan I.S.S. | No. 14, pp. 365, 366 | Adult chest<br>Children                   |
| Schopper             | Contax or Leica F/1.5-F/2   | Ilektoplan (Auer)   | 15 times reduction                         | 80 cm.         | 80         | 100        | 0.12                         | Agfa Fluorapid                       | No. 13               | Chest                                     |
| Böhme                | 16 mm.  | Yellow-green fluorescent  |  |                | 80         | 50-50      | 24 to 32 frames per second   |                                      | No. 16a              | Cat thorax                                |
| Stewart and Ghiselin | Zeiss B-Biolar, F/0.85<br>5.5 cm. focal length 16 mm.<br>3 3/4 x 3 3/4<br>F/1.5 | Spectral cyanide yellow green<br>Patterson Fluorazure (Photo-Röntgen) |  |                | 100        | 110        | 16 frames per sec.           | EK Super X                           | Nos. 16 and 33       | Heart                                     |
| Files                |   |   |  | 48 in.         | 55-80      | 400        | 1/10 adults<br>1/20 children | High Speed X-ray                     | No. 34               | Chest                                     |
| Steps                | Zeiss Biolar F/1.4 35 mm. or 2 1/4 x 3 1/4                                      | Neosol or Super-Astral  | 100 cm. (35 mm.)<br>77 cm. (2 1/4 x 3 1/4) | 150 cm.        | 50-57      | 500 to 600 | 0.22                         | Agfa Isopan I.S.S.                   | No. 35               | Chest                                     |



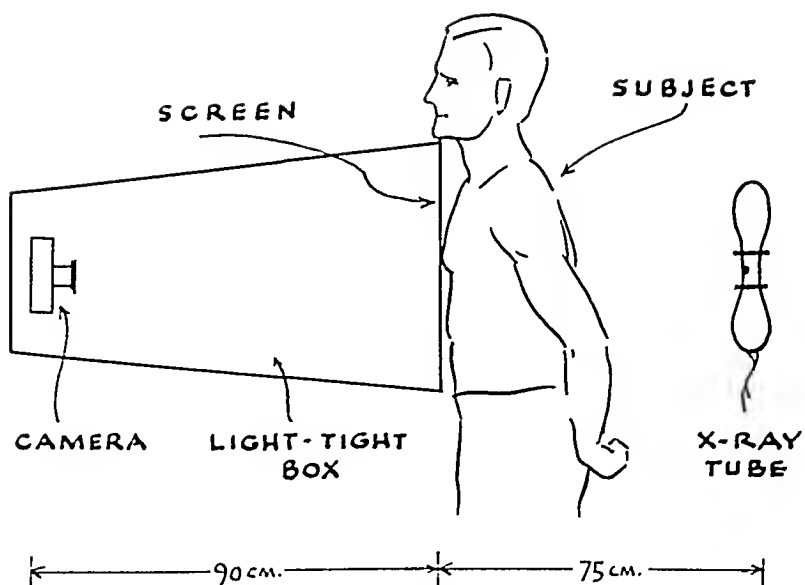


FIG. 3A.

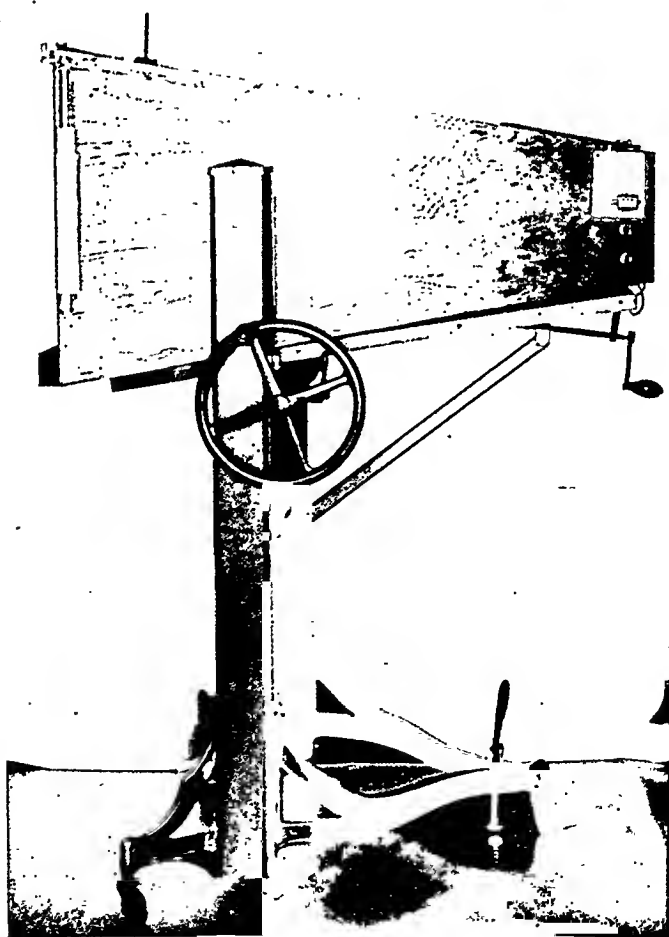


Fig. 3B.

tion is that any equipment of 150 Ma. capacity in conjunction with a roentgen-ray tube of the rotating anode type, capable of operating at 100 kv. loads, is adaptable to the process. For a guide in exposure, Table III has been prepared giving the data recommended by various workers.

## PROJECTOR FOR VIEWING FLUOROGRAMS

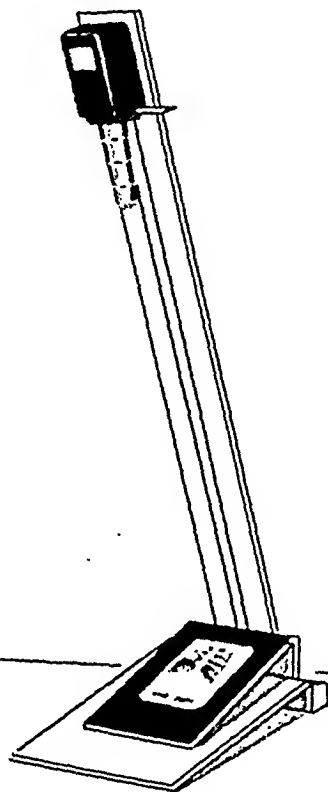


Fig. 1.

For the processing of the film, standard roentgen-ray developing and fixing solutions are satisfactory. The film can be looped around suitable racks if long lengths are employed, or small 35 mm. film developing tanks can be used for standard 5½ feet, 35 exposure lengths.

The fluorograms are projected when viewed, and for this purpose any number of adequate 2 by 2 inch slide projectors are available. A diagram of the projection arrangement used by Hirsch is shown in Fig. 1.

A convenient magnification of the fluorogram does not yield an image as large as the screen image, but if a 6.8 times enlargement is used, the size will be 15 per cent smaller than half size. For the average chest, this 15 per cent undersize just corrects for the distortion introduced by the use of a 30 inch target-screen distance and accordingly gives a true half-size view.

### CONCLUSIONS

The advantages of fluorography in medical roentgenography are, chiefly, simplicity and economy in conducting mass examinations. The economy comes

from the lower cost of small-sized film and subsequent economies in developing and filing. The cost of the film is approximately 2 per cent of that of 14 by 17 inch roentgen-ray film. Perhaps the best discussion on the economy and also on the advantages of fluorography that has been presented is contained in the article by Hirsch,<sup>29</sup> to which reference can be made. A vital comparison between fluoroscopic examination and the fluorographic method made by Hirsch is that 350 fluorograms can be read an hour while only 250 fluoroscopic examinations can be made a day by one person. Not only is the process more rapid than direct examination, but it is more dependable, and in addition furnishes a permanent record. Of course, fluorograms cannot compete with large-sized radiographs with respect to image detail and general applicability. However, fluorography certainly has its place in preventive medicine. It is a process with which every clinical photographer should become acquainted.

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# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**STERNAL MARROW, Changes During the First Week of Life. Correlation With Peripheral Blood Findings, Shapiro, L. M., and Bassen, F. A. Am. J. Med. Sc. 202: 341, 1941.**

Peripheral blood studies and sternal puncture examinations were made on 35 normal full-term infants in the first twenty-four hours of life and again one week later.

The peripheral blood findings agreed with those of many other investigators. The authors noted initial high levels of red blood cells, hemoglobin, white blood cells, and reticulocytes. There was a moderate decrease in red blood cells and hemoglobin, a sharp fall in white blood cells and reticulocytes, and a change in the differential from myeloid to lymphocytic predominance at the end of the week.

The most striking finding in the bone marrow was a marked drop in the erythroid elements at the end of the first week of life. This finding, reflected in the peripheral blood by the decreasing reticulocytes, is evidence of curtailed production of new red blood cells. Consequently, the authors offer the idea that the fall in red blood cells and hemoglobin, which takes place after birth, is primarily the result of physiologic disintegration of the superabundant red blood cells carried over from fetal life in the presence of diminished erythropoiesis.

**CEREBROSPINAL FLUID, Protein Values Determined by Tyrosine Equivalent Method, Marron, T. U. Am. J. Med. Sc. 202: 330, 1941.**

Total proteins have been determined by the tyrosine equivalent method on 550 cerebrospinal fluids taken from persons in health and disease.

The upper limit of normal values has been found to be 46.0 mg. per 100 c.c. The ranges for pathologic values agree well with generally accepted figures in the literature.

The average protein values for females is less than that for males.

Dehydration does not appreciably affect the protein concentration.

**TUBERCLE BACILLI, Demonstration of, by Fluorescence Microscopy, Richards, O. W., Kline, E. F., and Leach, R. E. Am. Rev. Tuberc. 44: 235, 1941.**

## Procedure:

1. Make direct smears of sputum, or of sputum concentrated on glass slides, and fix by heat in the usual manner.
2. Stain for two or three minutes with a solution of 0.3 per cent auramine in 3 per cent carbolic acid solution at room temperature.
3. Wash with water.
4. Decolorize with a solution of 0.5 per cent concentrated hydrochloric acid and 0.5 per cent sodium chloride in 70 per cent alcohol for one minute, pour off, and add fresh decolorizer for two additional minutes.
5. Rinse, dry, and examine.

Smears should be made preferably with fresh sputum. If a preservative is desirable, phenol will be more satisfactory than cresol, which reduces the number of organisms and causes an interfering fluorescence. Autoclaving will greatly reduce the number of organisms seen.

This staining technique is neither a painstaking nor a critical one. Almost any concentration of auramine will give the desired result; as little as 0.05 per cent of dye may be used. The concentration of phenol may be reduced. Satisfactory results have been

obtained with 0.5 per cent auramine in 2 per cent phenol. The stain originally recommended was 0.1 per cent auramine in 5 per cent phenol. The auramine is dissolved with gentle heat, but the stain becomes cloudy on cooling. Cloudy solutions are just as satisfactory as clear ones, but should be shaken just before use. The stain, after a number of weeks, gradually loses some of its fluorescent properties. Smears of known positive sputa should be stained and examined occasionally as a check on the keeping qualities of the dye.

Almost any of the standard decolorizing agents mentioned for decolorizing tubercle bacilli, such as 3 per cent hydrochloric acid, 5 per cent nitric acid, or even 25 per cent sulfuric acid, may be used. These stronger reagents require more careful timing; most of them decolorize in from twenty to thirty seconds. The slower, three-minute period decolorizer is recommended.

The newly designed equipment for fluorescence demonstration of acid-fast organisms has the following advantages over older methods for examining smears of sputum and other material for the presence of tubercle bacilli:

(a) The method stains more acid-fast bacteria than does the Ziehl-Neelsen technique.

(b) Examinations can be made with high dry objectives that cover fields which are larger than those covered by oil-immersion lens as well as being simple and more convenient for use.

(c) A greater contrast is obtained between the stained organism and other material present on the slide.

**MONONUCLEOSIS, Infectious, Observations on, Warren, E. W. Am. J. Med. Sc. 201: 483, 1941.**

The authors present evidence of the following phenomena to be found in infectious mononucleosis: Forssman heterophile antibodies are probably increased prior to the development of the heterophile antibodies of this disease and possibly are essential for their appearance. The typical lymphocytes of infectious mononucleosis are frequently found in disease entities in which a positive Paul-Bunnell has not been obtained. Only when the proportion of these abnormal cells is considerable does one find an elevated heterophile titer.

**CL. WELCHII, Rapid Identification of, by the Nagler Reaction, Hayward, N. J. Brit. M. J., May 31, p. 811, 1941.**

The production of a turbidity in human serum by the toxins of growing *Cl. welchii* (Nagler reaction) is a valuable aid to the rapid identification of the bacillus for diagnostic purposes. Excellent results are obtained with a mixture of equal parts of human serum and Fildes broth incubated anaerobically. The seeding of a single *Cl. welchii* colony into such a mixture produced a strong reaction in 80 per cent of the tests after one to two days' incubation. The reaction is specifically inhibited by antitoxin. Feeble reactions are not unequivocally indicative of *Cl. welchii*, since they are given by certain other species of anaerobic spore-bearer. Human serum-Fildes agar provides a means of detecting *Cl. welchii* on plates within twenty-four hours of taking a swab.

**FUSOSPIROCHETOSIS, Recovery of the Causative Organism From the Blood, Williams, R. H. Arch. Int. Med. 68: 80, 1941.**

The recovery of fusiform bacilli and spirochetes, of the type found in Vincent's angina, in cultures of the blood of 2 patients is recorded. Fusospirochetes were recovered eleven times from one patient and once from the other. Successful subcultures of the organism were made repeatedly. The organisms were slightly pathogenic for rats.

In the 2 patients fusospirochetemia was associated with an illness characterized by the sudden development of fever, prostration, headache, myalgia, and migratory arthritis. In one patient splenomegaly developed, with jaundice, a hemorrhagic eruption, and acute pleuritis. The source of infection is considered to be the tonsils in one instance and the bite of a rat in the other.

**EOSINOPHILIA, Extreme, and Leucocytosis, Bass, M. H.** *Am. J. Dis. Child.* 62: 68, 1911.

Three cases in which the disease was characterized by general adenopathy, leucocytosis, and extreme eosinophilia that occurred in childhood are reported.

In one case the patient had roentgenographic evidence of miliary pulmonary infiltrations, which persisted for several years but finally disappeared.

The leucocytosis, and especially the eosinophilia, remained long after the fever had disappeared. The course of the disease is chronic, but it may end in complete recovery.

In one case death occurred from intercurrent illness during the first year of observation. In the second case the patient was kept under observation for several years and finally recovered completely. In the third case, in which the patient is still being followed, there was a febrile period, but the child is now apparently in good health; the total leucocyte count has returned to normal, but a marked increase in eosinophilic cells still persists.

Data from 4 cases, reported from Havana, in which the disease resembled that in these cases are cited. The 7 cases resemble each other closely and appear to be examples of a clinical entity.

The relation of this syndrome to other pathologic states in which there are extreme leucocytosis and eosinophilia is discussed, and it is shown that the syndrome fails to fit into the category of any of the previously described conditions. Although the cause of the syndrome is unknown, it is most probably some type of chronic infection.

**SULFANILAMIDE, Erythrocyte Fragility Changes Produced by, Antopol, W., Goldman, L., and Sampson, W. L.** *Am. J. Med. Sc.* 202: 163, 1911.

The administration of sulfanilamide to rats results in an increased resistance of the red blood cells to hemolysis in hypotonic saline solution, and in splenomegaly with the histologic picture of active hyperemia such as seen in hemolytic anemia.

**CORONARY ARTERY OCCLUSIONS, Incidence and Localization of, Schlesinger, M. J., and Zoll, P. M.** *Arch. Path.* 32: 178, 1911.

More than one-half of the points of occlusion in the coronary arteries are overlooked by ordinary dissections.

Most zones of occlusion of the coronary arteries are less than 5 mm. in length and are, therefore, easily overlooked.

Occlusions are as numerous in the right coronary artery as in the left descending coronary artery.

There is no relationship between the manner of branching of the coronary arteries and the localization of occlusions therein.

The majority of coronary artery occlusions are found within 3 cm. of the mouths of these vessels.

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## ITEM

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### Urology Award

The American Urological Association offers an annual award "not to exceed \$500.00" for an essay (or essays) on the result of some specific clinical or laboratory research in urology. The amount of the prize is based on the merits of the work represented, and if the Committee on Scientific Research deem none of the offerings worthy, no award will be made. Competitors shall be limited to residents in urology in recognized hospitals and to urologists who have been in such specific practice for not more than five years.

Essays shall be in the hands of the Secretary, Dr. Clyde L. Deming, 789 Howard Avenue, New Haven, Conn., on or before April 1, 1942.



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## CLINICAL AND EXPERIMENTAL

### SYSTEMIC HISTOPLASMOSIS\*

#### SYSTEMIC HISTOPLASMOSIS DIAGNOSED BEFORE DEATH AND PRODUCED EXPERIMENTALLY IN GUINEA PIGS

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THE discovery by Darling<sup>1</sup> in 1906 and the subsequent report of three cases of histoplasmosis in Panama has been followed by the observation of seven cases of this fatal systemic disease in this country. We are recording here the eighth case of systemic histoplasmosis that has occurred in the United States.† Diagnosis was made from the blood smear and blood culture during life. The disease in a systemic form also has been transmitted to guinea pigs for the first time.

Between the years 1906 and 1909 Darling<sup>2</sup> reported three cases of a systemic parasitic infection, the causative organism of which he named *Histoplasma capsulatum*. In each case diagnosis was made following autopsy, and Darling, because of the clinical picture and the resemblance of the parasites to Leishman-Donovan bodies, believed it to be a tropical disease related to kala azar. Not until 1926 was the next case recorded, and this by Riley and Watson,<sup>3, 4</sup> was the first to be reported from North America. In the same year, Phelps and Mallory<sup>5</sup> reported a case of "histoplasmosis of the lung," but neither the disease nor the pathology of this case resembled that of systemic histoplasmosis. A fifth case was found in an American negro in 1931 and was reported by Crumrine and Kessel.<sup>6</sup> In all these cases the diagnosis was made only after autopsy, and attempts to culture the parasite were unsuccessful. Dodd and Tompkins<sup>7</sup> dis-

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†The report was written seven additional cases of systemic histoplasmosis have been reported by H. E. (Am. J. Trop. Med. 20: 693, 1940), reviewing the literature, records cases, four of which appear to be systemic.

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covered in 1934 the sixth case in an infant from Tennessee. The diagnosis was made in this case for the first time during life by the finding of the parasites in the monocytes of the blood. For the first time, likewise, the organism was successfully cultivated from material obtained at autopsy, and DeMonbreun<sup>3</sup> was able to produce the disease in monkeys by intravenous injection of cultures.

Subsequently, Agress and Gray,<sup>9</sup> Amolsch and Wax,<sup>10</sup> Shaffer, Shaul, and Mitchell,<sup>11</sup> and Clemens and Barnes<sup>12</sup> reported cases of systemic histoplasmosis in which diagnosis was established at postmortem by the finding of the parasites within the cells of infected organs. Culture of the parasite from the spleen was obtained in only one of these four cases, that reported by Clemens and Barnes.<sup>12</sup>

Clinically, systemic histoplasmosis, as described by Darling, is characterized by "splenomegaly, emaciation, irregular pyrexia, leukopenia, and anemia." Only in the case of Crumrine and Kessel was splenomegaly absent. The pathology has been described as "the invasion of endothelial cells in the smaller lymph and blood vessels and capillaries by enormous numbers of a small encapsulated organism (*Histoplasma capsulatum*), causing necrosis of the liver with cirrhosis, splenomegaly, pseudogranuloma of the lungs, small and large intestines, with ulceration of the latter, and necrosis of the lymph nodes draining the injected viscera."<sup>13</sup>

#### CASE REPORT

The patient, a 38-year-old negro man, was referred to St. Philip Hospital, on March 23, 1939, for diagnosis. His chief complaint was "weakness," a symptom which had become progressively more pronounced since it was first noticed in mid-December. A janitor in a real estate office, he had continued at work until the day before admission, in spite of an increasing weakness of his arms and legs, which steadily increased the effort required in the performance of his routine duties. He had had a loose cough with slight expectoration since January, and had noted profuse sweating, associated at times with the sensation of "chills." The sweats were noticeable especially at night, often being of a severity sufficient to make him get up and change his clothing. For two months previous to his admission to the hospital he had been unable to walk more than a city block without sitting down to rest because of dyspnea and weakness in the knees. His appetite had grown progressively poorer, and during the last week he had drunk only milk. Hoarseness also had been noted during the last week. The patient believed he had lost about 50 pounds in weight, and for three weeks had been troubled with a watery diarrhea.

Past history was nonessential, except for the observation that the patient, born in South Carolina, had lived in Richmond, Va., for the past twenty years and had had no known contacts with the tropics. His health had been good until the present.

**Physical Examination:** The patient's skin was hot and moist. His mucous membranes were pale. He was very cooperative but a marked hoarseness made difficult his response to questioning. His temperature was 102° F., pulse rate 108, and respiration, 22 per minute. Examination of the eyes revealed small, white, irregular areas surrounded by hemorrhages in both fundi. These were not unlike tubercles. The pharynx was reddened. On laryngeal examination there was seen to be edema of both arytenoids with hyperemic cords which did not approximate well. The right cord especially was roughened, but there were no ulcerations. Lymph glands in the axillary, cervical, submental, and popliteal areas were enlarged, soft, discreet, and nontender. Chest examination revealed moist râles in both bases posteriorly and bronchial breathing on the right, anteriorly, just below the nipple area. With the exception of a soft systolic murmur, which was heard over the apex and not transmitted, heart examination was negative. The blood pressure was systolic 124 and diastolic 60. The abdomen was negative; neither the spleen nor the liver was palpated. There were no alterations in reflexes. The admission impressions were miliary tuberculosis, bacterial endocarditis, and Hodgkin's disease.

**Laboratory Examination:** Urinalysis: specific gravity 1.013, trace of albumin, occasional pus cell and hyaline cast. Blood: red cells 2,540,000; hemoglobin 54 per cent; white cells 3,200; differential count: neutrophils, 68 per cent, lymphocytes, 28 per cent, monocytes 4 per cent. Bleeding and clotting times were normal. Blood chemistry: sugar 141 mg., nonprotein nitrogen 25 mg., calcium 7.8 mg., phosphorus 3.1 mg., total protein 4.7 mg., albumin 2.1, and globulin 2.6. Complement fixation and flocculation tests were negative. Spinal fluid, stool, and agglutination examinations were also negative.

Röntgenologic examination of the chest showed "irregular mottling on the right first and second interspaces anteriorly with moderate density of the hilum and trunk shadows on the right, suggesting definite, minimal tuberculosis of the right lung."

In the hospital the patient ran a septic type of temperature which was characterized by an irregular regularity. This ranged between 100 and 103 degrees, rising to the higher level between 4 and 12 P.M. and falling during the night and early morning, only to rise again in the late afternoon or evening. This occurred daily without exception until the final week of his illness, when the temperature fluctuated between 99 and 101 degrees with the higher level still in the afternoon.

The patient continued to grow weaker, and, when repeated sputum examinations were negative for the tubercle bacillus, the hematologic service was consulted because of the anemia.

Study of the blood smear showed no malarial parasites. However, because of the presence of an endothelial type of monocyte, one of us (H. L.) continued to examine the smear further, the search being finally rewarded by the finding of a large phagocytic cell containing numerous small bluish hyaline bodies which, at the time, were not identified. The report on this examination was: "Blood smear shows slightly regenerative, hypochromic, microcytic anemia with rare normoblasts. There is a left shift at the stab level and the neutrophils show slight toxic granulation with frequent Döhle bodies. An occasional 'blast' cell is seen and the platelets appear greatly reduced. On checking, these were found to number 122,000 (normal 500,000). Phagocytic monocytes are present and rarely one of these is seen to contain numerous small bluish hyaline bodies with dark staining nuclei. These measure  $2\mu$  to  $4\mu$  and vaguely resemble Leishman-Donovan bodies."

Sternal puncture was resorted to for further study of the blood. The bone marrow aspiration showed numerous monocytes filled with the parasites, and there were numbers of neutrophils containing from one to five organisms. The marrow was moderately erythroblastic and contained a number of plasma cells. Megakaryocytes were notably decreased. In addition to the parasites there were phagocytosed red and white blood cells in the large endothelial cells, many of which were binucleated.

Following this finding, an axillary lymph node was removed. This showed a chronic lymphadenitis with proliferation of the reticulum, the reticulum cells, under oil immersion, being engorged with numerous inclusion bodies which possessed a pale peripheral halo and a faint oval basophilic center.

A previously negative blood culture was re-examined and a fresh culture was taken. These were examined for fungi and found to be positive for *Histoplasma capsulatum*. In the meanwhile daily blood smears continued to show the parasites in increasing numbers. While at first the parasites had been observed only in the monocytes, as the disease progressed the neutrophils were found to contain them. There was a steady increase in toxic degenerative granulation of the leucocytes, as normoblasts and myelocytes began to appear in greater numbers, and the patient, becoming gradually weaker, died in stupor on April 24, 1939.

#### POST-MORTEM EXAMINATION

(Abstract from Protocol)

Externally the only finding of interest was that of a moderate swelling of lymph nodes in the axilla, groin, and neck. No skin lesions of any type were noted.

**Thorax:** The right pleural cavity contained approximately 500 c.c. of clear straw-colored fluid. There was no effusion into the left pleural cavity or the pericardial cavity.

**Lungs:** The base of the right upper lobe in its lateral aspect showed a depressed stellate grayish-white area, measuring approximately 0.5 cm. in diameter in its central portion. Sections through this lesion showed a roughly triangular area slightly raised, measur-

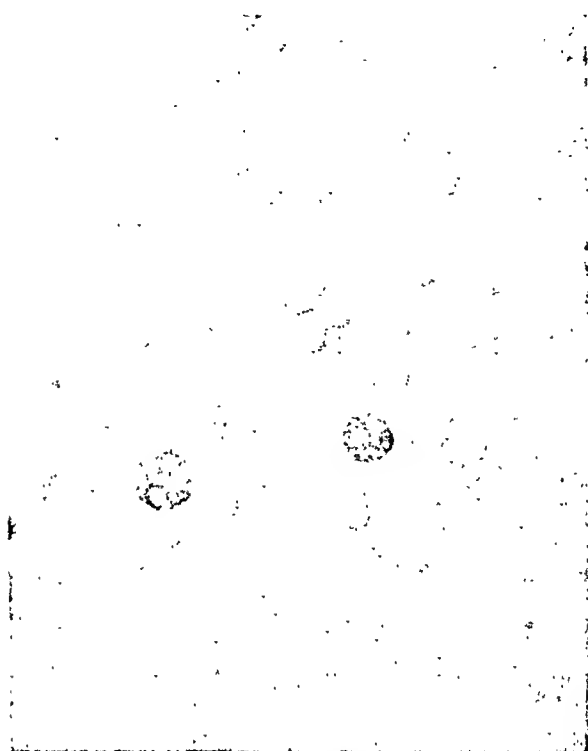


Fig. 1.—Peripheral blood showing parasites in polymorphonuclear cells.

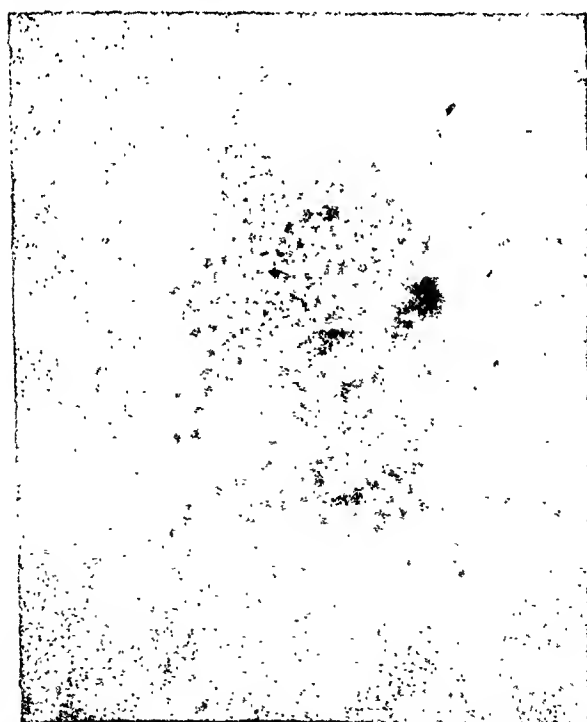


Fig. 2.—Large binucleated endothelial cells containing numerous parasites. Sternal bone marrow.

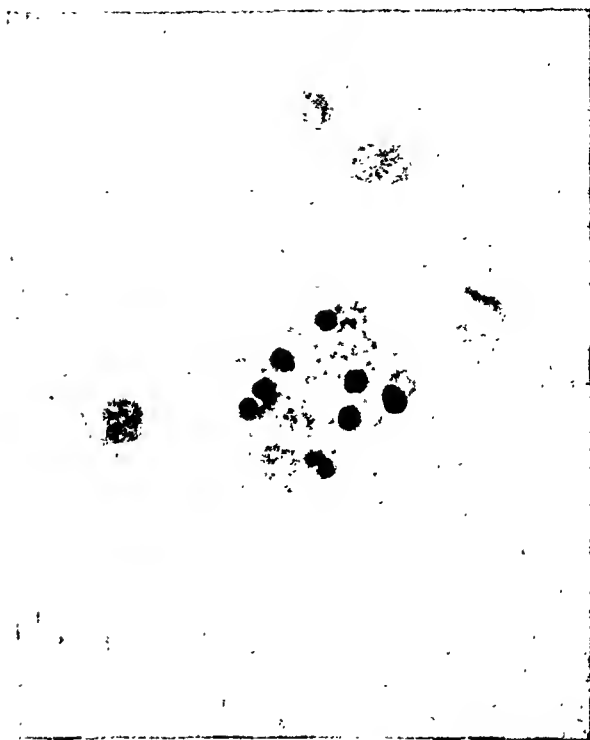


Fig. 3.—Endothelial cell from bone marrow puncture is shown with engulfed normoblasts and parasites.



Fig. 4.—Numerous endothelial cells with parasites seen in parts of smear from sternum.

ing about 2 cm. at its pleural base and 3 cm. from the base to its apex near the hilus. This area of infiltration was composed of discrete and frequently conglomerate nodules, measuring from the size of a millet seed to 3 mm. in diameter. They were situated for the most part peribronchially, but did not show rosette formation or any other distinct pattern of distribution. On cut surface the nodules were raised, grayish white in color and partially caseous in their center. The margins of the larger confluent areas of grayish-yellow necroses were firmer and presented a somewhat gritty sensation when scraped with a knife. The intervening tissue was slightly depressed beneath the cut surface with grayish-white prominent bands. There was abundant anthracosis in this area. No abscesses were seen. The remaining parenchyma of both lungs was normal. The hilar lymph nodes were moderately enlarged and contained carbon pigment; they failed to disclose distinctly visible nodules or areas of caseation.

**Spleen:** The spleen weighed 500 Gm. and measured 11 by 11 by 7 cm. It was firmer in consistency than normal. The pulp did not scrape away on the knife edge. The follicular and trabecular structures were obscured.

**Kidneys:** Each kidney weighed 200 Gm. and measured 10 by 7 by 4 cm. Their surfaces were very finely granular. The cut surface was very moist. There was an eversion of the cut edges. The corticomedullary demarcations were markedly obscured. The architecture of the medulla and cortex proper appeared to be normal.

**Liver:** The liver weighed 2,100 Gm. Its dimensions were 27 by 18 by 10 cm. Grossly it presented no abnormalities.

**Intestines:** The lower 45 cm. of the ileum, the cecum, and the ascending colon showed scattered mucosal nodules, measuring from 2 to 5 mm. in diameter, raised about 2 mm. above the surface. The smaller nodules were grayish white. On sectioning they were firm and solid. The larger ones showed central areas of caseation. In the cecum and ascending colon were found numerous areas of ulceration, mostly round in shape. Their margins were sharp but ragged and surrounded by a hemorrhagic zone. They did not seem to be undermined. The ulcers measured from 1 mm. to about 5 mm. in diameter.

**Lymph Nodes:** The mesenteric lymph nodes were moderately enlarged. The para-aortic lymph nodes and lymph nodes of the liver hilus were markedly enlarged, matted together in irregular masses adherent to the adjacent tissues. They were firm to palpation and on section showed irregular well-defined areas of yellowish necrosis.

**Vertebral Bone Marrow:** Showed grossly no abnormalities.

#### MICROSCOPIC EXAMINATION

**Organisms:** Characteristic organisms of *Histoplasma capsulatum* were found in the lungs, spleen, kidney, intestines, liver, pancreas, lymph nodes, and bone marrow. The organism itself was usually round, or slightly oblong, in shape, and measured approximately  $2\mu$  to  $4\mu$  in diameter. In both the ordinary hematoxylin-eosin stain and in Goodpasture's stain the external membrane was nonstaining and doubly refractile. The internal structure of the organism was variable. It contained a clear vacuolated nonstaining cytoplasm and a bluish-stained chromatin material, the arrangement of which varied greatly. In some of them the chromatin material was finely granular and found in the center of the body; in some it was situated at one or both poles in the form of small and larger round masses; in others these unipolar or bipolar masses were more crescentic in shape and partially encircled either one or both poles. In still others they seemed to encircle the periphery entirely. In all bodies the remaining portion of the organism was composed of a nonstaining cytoplasm which was negative for both Ziehl-Neelsen and fat stains. For the most part the organisms were found ingested in cells, but occasionally they were found free, singly and in groups.

**Typical Lesion:** The typical lesion produced in the various organs by *Histoplasma capsulatum* was a rather characteristic nodular granuloma resembling a tubercle or a gumma. The nodules varied in size from  $15\mu$  to  $20\mu$  to several millimeters in diameter. The smaller were circular in outline, the larger appeared to be confluent, disclosing serpiginous borders or a maplike outline. The central portions were composed of eosinophilic staining, partially granular, unidentifiable material. Some foci of necrosis contained many nuclear fragments.

In many of them, this central area of necrosis still revealed a shadowy outline of the underlying structures. Capillaries were found to be coursing through the entire nodule and were often seen to be patent and filled with blood, even in the center of the necrosis. The central necrotic areas frequently disclosed the structure of elastic membranes still completely or partially intact. The alveolar septa and vessels could easily be recognized within the areas of necrosis by elastic tissue stains. At the periphery the necrotic areas shaped themselves into ill-defined faintly staining "ghosts" of markedly engorged mononuclear cells. Still more peripherally the large monocytes were well preserved. They contained ingested material which was composed of large numbers of closely packed *Histoplasma capsulatum* organisms. About the periphery of the nodules were scattered plasma cells, lymphocytes, and a few polymorphonuclear cells. Giant cells and epithelioid cells were absent.



Fig. 5.—Yeastlike forms of *Histoplasma capsulatum* from a six-day-old culture on sealed blood agar slant at 37° C. Wet mount (X700).

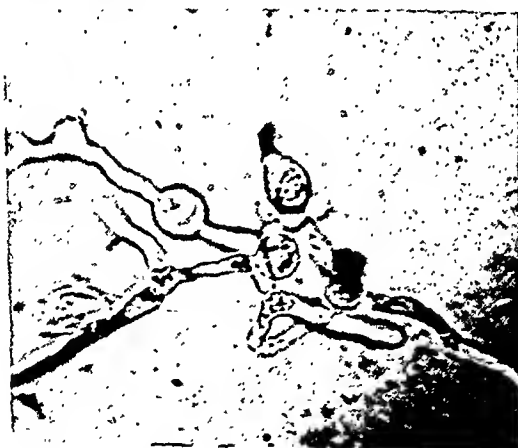


Fig. 6.—Hyphae developing from yeastlike form in semisolid culture media containing fresh blood at 37° C. Wet mount (X700).

*Lungs:* Sections through the lesions at the base of the upper lobe of the right lung showed many of the typical granulomas described. Here they were mostly confluent. In one area a cytoplasmic mass was encountered which resembled a Langhans' giant cell: vesicular nuclei were grouped around a pink-staining cytoplasm. It could not, however, be definitely identified as a foreign body giant cell. In addition to the above-described typical lesion there was a marked polymorphonuclear cell infiltration in a diffuse form in the

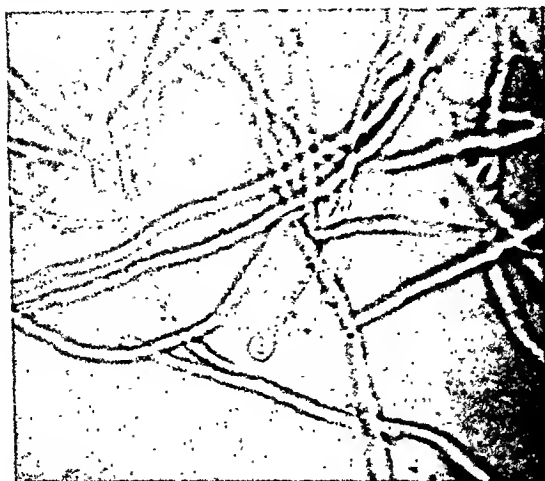


Fig. 7.—Early mycelial type of growth in same media as Fig. 6 at room temperature. Wet mount ( $\times 700$ ).

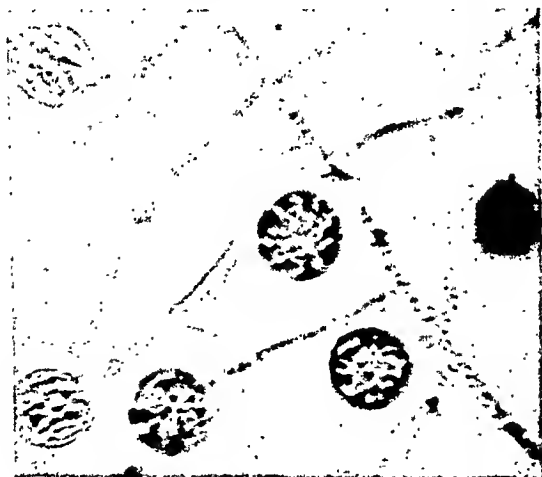


Fig. 8.—Development of mycelial form on dextrose agar plates at room temperature, showing enlarging arthrospores. Wet mount ( $\times 700$ ).

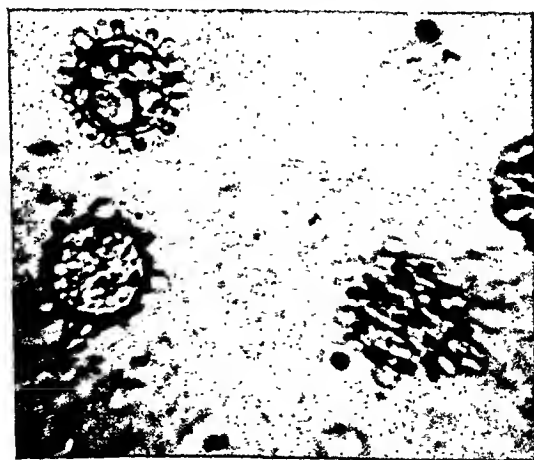


Fig. 9.—Further development of arthrospores showing the tuberclelike protrusions. Wet mount ( $\times 700$ ).



interalveolar septa. There were also discrete areas of dense infiltration with polymorphonuclear cells, which showed a liquefactive necrosis of the underlying tissues. These possessed all the characteristics of a true, though small, abscess. Marked interstitial infiltration with lymphocytes and plasma cells was found in the vicinity of the above-described granulomatous nodules in the interalveolar septa. There was extensive fibrous scarring throughout. A number of large discrete areas were encountered which resembled old-healed conglomerate tubercles. In fact, from a histologic point of view, they could not be distinguished from the latter. It is uncertain whether or not such foci should be looked upon as a healing phase of the granulomas caused by *Histoplasma capsulatum*.

Vessels were involved in the inflammatory processes. Arteries of various sizes were seen with proliferating endarteritis and complete obstruction of their lumina. The granulation tissue in many areas involved medium-sized and smaller veins. Elastic tissue stain disclosed that all layers of the wall were infiltrated by a variety of small round cells. The elastic lamellae, in particular those of the adventitial layers, were "teased up," split apart, and partially disintegrated. The process encroached upon the media and resulted in disintegration of the wall and segmental disappearance of elastic fibers. Many veins showed proliferation of subintimal cells with formation of intimal pads. In some of these the granulation tissue completely occluded the lumen.

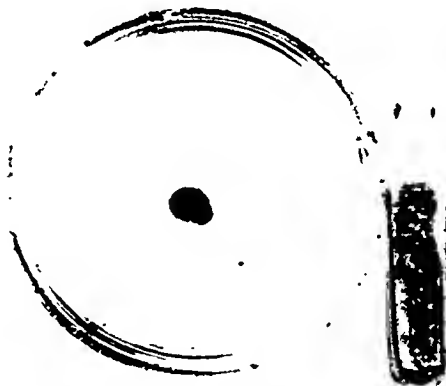


Fig. 10.—Three weeks' old culture on Sabouraud's dextrose agar at room temperature. The aerial hyphae which give the colony its fluffy appearance are plainly visible. Wet mount ( $\times 0.5$ ).

**Spleen:** The spleen showed many minute discrete nodules of the typical type. Polymorphonuclear cell reaction, however, within or in the vicinity of these granulomas was rather scarce. The sinusoids were dilated and contained large monocytes and desquamated reticulum cells, both of which were markedly engorged with organisms. There was diffuse hyperplasia of the reticulum which was best seen in silver stains.

**Pancreas:** The pancreas showed a slight diffuse lymphocytic and plasma cell infiltration of the somewhat edematous interstitial connective tissue. There were also scattered macrophages that contained the ingested organisms. Characteristic nodules were not encountered.

**Kidneys:** The kidneys did not show specific nodules. The organisms, however, were occasionally found in large cells in the glomerular tufts. It could not be determined whether these cells were endothelial or epithelial. The interstitial tissue of the cortex showed edema and a moderate infiltration with plasma and round cells. Occasional polymorphonuclear cells were also encountered.

**Liver:** The liver contained many minute and larger typical *Histoplasma capsulatum* nodules. They bore no special relation to the liver lobules, some being found in the portal areas, others midway between the center and the periphery of the lobules, and still others

occupying most of the lobule. In addition, Kupffer cells throughout the entire liver substance were greatly increased in size and contained the organisms. Otherwise the liver was essentially negative.

*Intestines:* The ulcers about the ileocecal region were rather superficial, and, as a rule, did not extend deeper than the submucosa. The edges were rather sharp, not undermined. The floor and margins were composed of necrotic tissue with nuclear fragments and "ghosts" of large mononuclear cells with phagocytosed organisms. Beneath the floor was found a layer of nonspecific granulation tissue that contained many mononuclear macrophages with ingested organisms, a variety of larger and smaller round cells, fibroblasts, and capillaries. Polymorphonuclear cells were found abundantly within this granulation tissue. The involvement of the vessels was of the same type as that described in the lungs.

*Lymph Nodes:* They contained many typical granulomas already described. Reticulum cells throughout were very conspicuous, being greatly engorged with organisms. The follicles were small and widely separated by diffuse hyperplastic reticulum. Polymorphonuclear cells were scarce. Many veins in the periphery were engorged with macrophages that contained the organisms. Periendophlebitis, as described in the lung, was likewise encountered in the vicinity of the caseous granulomas.

*Bone Marrow:* Rather actively functioning, the bone marrow contained numerous large mononuclear macrophages and reticulum cells engorged with the organisms.

*Brain:* Permission for examination of the brain was not given, and it was, therefore, not possible to examine the eyes.

The remaining organs of the body were essentially negative.

#### MYCOLOGIC STUDIES

The clinical symptoms of histoplasmosis are so ill-defined that only one of the ten cases published previously has been diagnosed before the death of the patient. In this case, presented by Dodd and Tompkins,<sup>7</sup> the typical yeastlike bodies of *Histoplasma capsulatum* were found in the smears of the blood. Later, the fungus was also cultivated from the blood by DeMoubrun.<sup>8</sup> Clemens and Barnes<sup>12</sup> have also reported finding the parasite in a blood culture taken on their case and examined one month later after diagnosis was established at autopsy. Since the ante-mortem diagnosis of this infection rests, at the present time, upon these findings, we are presenting the cultural methods used in obtaining the parasite from this case of systemic histoplasmosis.

The blood culture media utilized for routine work in the laboratory and which we used in this study is prepared as follows: Veal infusion broth containing 0.3 per cent dextrose, 0.5 per cent sodium citrate, and 0.2 per cent agar adjusted to pH 7.4 is sterilized in 40 c.c. amounts in small Erlenmeyer flasks. Usually 10 c.c. of blood are taken from the patient and inoculated directly into the flask. Incubation is carried out aerobically or anaerobically, as the case may warrant, at a temperature of 37° C.

The first blood culture was received from the patient four days after admission to the hospital, and approximately fourteen weeks after the onset of symptoms. This culture was found to be contaminated and was discarded at the end of four days' incubation. A second blood culture was received three days later. There were noticed in this flask at the end of twelve days' incubation numerous yeastlike bodies and a few scattered mycelial threads. One of us (H. I.) had found similar bodies somewhat earlier in smears prepared from the peripheral blood. These bodies were always found within the cells, particularly within the cytoplasm of the large monocytes. The yeastlike cells in the blood culture media tended to adhere to one another after division and were

often found in small clusters, particularly in wet preparations. One or two dark granules were visible within the otherwise clear cytoplasm of these unstained cells. When stained by Wright's method, the organisms were almost identical in appearance to those occurring in the tissue cells. They appeared as oval, occasionally round, cells,  $2.7\mu$  to  $3.6\mu$  in their longest diameter, exclusive of the capsule surrounding them. In this respect they were slightly larger than the parasites within the cells, the majority of which were between  $1.8\mu$  and  $2.7\mu$ , although infrequently larger cells measuring up to  $3.6\mu$  could be found. Within the parasite was a dark staining chromatin-like mass placed either at the larger end or in a semilunar arrangement at the end and along the side walls of the cells.

On the same day that this finding was made, a third specimen of blood was taken in citrate solution. When this was cultivated on Sabouraud's dextrose agar plates, fungi again were isolated.

Subcultures of the first blood culture showing growth were made in duplicate on Sabouraud's dextrose agar, dextrose extract agar, brain veal agar, and sealed brain veal blood agar slants. One set of slants was incubated at  $37^{\circ}\text{C}$ . and the other set at room temperature. Growth first appeared in the subcultures on Sabouraud's dextrose agar and dextrose extract agar kept at room temperature. Within five to six days small white colonies with a downy surface were visible. They slowly increased in size, with the production of white, feathery, aerial hyphae, arising from a base which became brown in color as the colony aged. The colonies adhered tenaciously to the surface of the media and were removed with difficulty. Similarly growth appeared on the plain brain veal agar slightly later, but at no time was growth on this media as heavy or as rapid as that on media containing dextrose.

Wet preparations of material from these colonies showed branched, hyaline, septate mycelium,  $2\mu$  to  $5\mu$  in diameter, within which were seen many dark, dancing granules. Developing arthrospores,  $8\mu$  to  $13\mu$  in diameter, were visible. They occurred on the ends of branches or from stalks along the hyphae. These smooth-walled spores also contained numerous sporelike bodies which Moore<sup>14</sup> considered to be endospores. The studies of Howell<sup>15</sup> have shown these larger cells to be true aleurospores. Occasionally in old cultures one could see tuberculate spores, pyriform in shape.

On sealed blood agar slants and in semisolid culture media containing fresh blood incubated at  $37^{\circ}\text{C}$ . the tendency was for the fungus to grow in the yeastlike stage. Cells somewhat larger than those seen in the tissues formed. These observations were similar to those of DeMonbreun,<sup>8</sup> who first determined the two forms of growth for this organism. The first, the yeastlike form, occurs in the body and on blood agar slants. He used the growth from these slants to reproduce the disease experimentally in monkeys. The second, the mycelial stage that we have described above, occurs on media incubated aerobically at room temperature. Intermingled with the yeastlike forms were larger cells from which hyphae appeared to be developing. The gross appearance of the growth on sealed blood agar slants was that of dull, grayish-yellow colonies, adhering well to the media. The surface of the colonies was smooth with slight tendency to the formation of aerial hyphae.

## PRELIMINARY ANIMAL EXPERIMENTS

Mice. Ten mice were inoculated intraperitoneally with amounts varying from 0.5 c.c. to 1.0 c.c. of a saline suspension of the six days' growth from the sealed blood agar slants. Although there was some indication of infection in two mice, the results with these animals were unsatisfactory, perhaps due to their relative insusceptibility to infection with the fungus.

Rabbits. Two rabbits were injected intravenously with 1.5 c.c. each of a similar suspension to that used in mice. Seven weeks later the animals were killed and autopsied. The organs were normal on gross appearance. Smears prepared from various tissues failed to show the *Histoplasma capsulatum* bodies.

Guinea pigs. One guinea pig was inoculated intraperitoneally with 1.0 c.c. of the original growth in the blood culture media. This culture had been incubated for two weeks at the time it was used for the animal inoculation. The pig gradually lost weight, and at the end of seven weeks was greatly emaciated. It was killed at this time and examined for evidence of infection. Lesions typical of those found in histoplasmosis were present.

There was a collection of fluid in both the peritoneal and pleural cavities. The spleen was enlarged to approximately three times its normal size. The inguinal lymph nodes were enlarged. The lesions in the lung partially involved the pleura and gave rise to a local fibrinous pleuritis. Smears made from the peritoneal fluid, spleen, lymph nodes, lungs, liver, and bone marrow showed many histoplasma bodies present both free and within the cells.

Histologic examination. Small granulomas were seen in the lungs, liver, and spleen. *Histoplasma capsulatum* organisms were noted in these organs and also in the lymph nodes and bone marrow. The lesion was essentially similar to that described in the patient.

*Histoplasma capsulatum* was grown from tissue taken from the lungs, spleen, and lymph nodes.

## COMMENT

The hematologic aspects of our case are of particular interest, for if the diagnosis is to be made before autopsy it must be from a study of the blood and blood culture. Once one has seen these large, vacuolated endothelial phagocytes with wavy, pale staining, light blue cytoplasm engulfing numbers of the parasites, one should be able to recognize them thereafter without difficulty. The disease may be more common than has been supposed, and the finding of an anemia with leucopenia in a weakened, emaciated patient who is found to have a septic temperature should lead one to search the blood smear for these parasitic-laden monocytes.

Presence of the *Histoplasma capsulatum* in the neutrophils has not been noted in other cases reported. In the terminal phase these were noted in increasing numbers, the polymorphonuclear cells containing from one to five parasites. The further observation that the organisms, as in kala-azar and malaria, appear in great numbers in the bone marrow makes sternal puncture valuable as a diagnostic approach in suggestive cases.

The stage of the disease in which the parasite may first be found in the blood in human beings is not at present known. As sternal puncture, performed the

same day the organism was found in the blood only after prolonged search, showed parasites in the bone marrow in great numbers, this should prove a valuable aid in early diagnosis. Blood culture at this time also was positive.

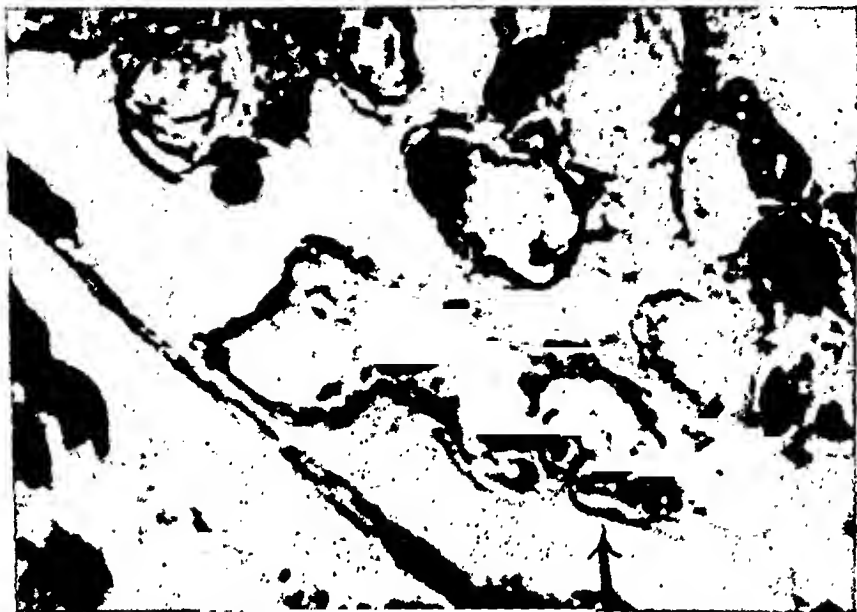


Fig. 11.—*Histoplasma capsulatum* organism in an endothelial cell in a glomerulus of the kidney ( $\times 2,500$ ).

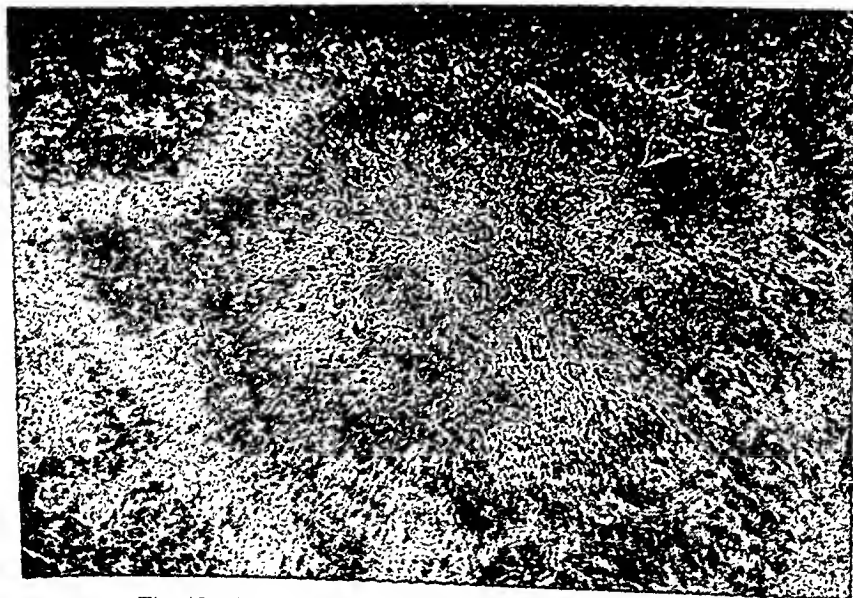


Fig. 12.—Large tubercle-like granuloma in the lung ( $\times 25$ ).

Dodd and Tompkins<sup>7</sup> felt that the anemia of histoplasmosis was to be attributed in part to the phagocytosis of red blood cells. While phagocytosis must undoubtedly play a part, the leuco-erythroblastic type of blood smear and the numbers of large monocytes found in the bone marrow would suggest

that the anemia is largely due to crowding of the marrow by numbers of these large monocytes.

The tubercle-like lesions with hemorrhage in the fundi have not been noted in the other reported cases. It is unfortunate that post-mortem verification of these lesions was not possible.



Fig. 13.—Elastic tissue stain, lung. Granulation tissue breaking through the wall producing subintimal proliferation ( $\times 150$ ).

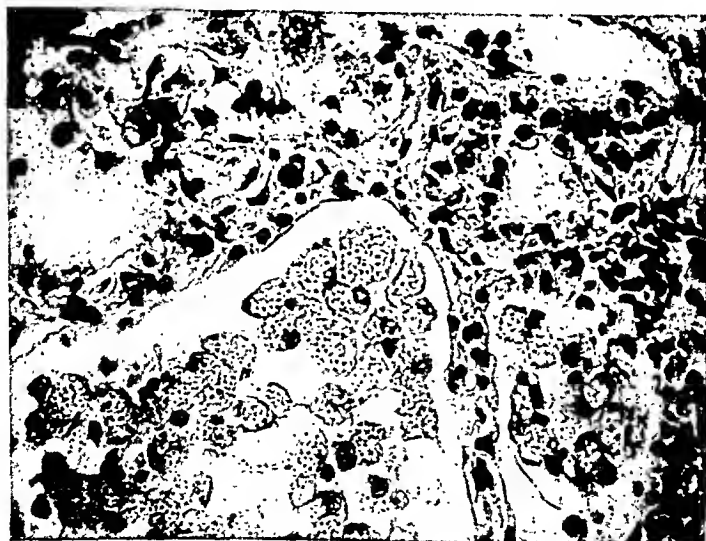


Fig. 14.—Alveoli of the lung containing large endothelial cells filled with the parasites ( $\times 360$ ).

*Histoplasma capsulatum* can be grown with comparative ease on a variety of media, using either fresh or citrated blood from the patient. However, flasks of liquid or semisolid media, such as we have used, are most desirable because of the larger amounts of blood that can be inoculated.

Initial growth is slow in the blood culture media incubated at 37° C. The yeastlike forms that develop under these conditions may not be numerous enough to be easily seen in wet mounts or stained preparations until one or two weeks have elapsed. When stained by Wright's method, the young cells are in appearance very similar to those seen in blood smears from the patient. The dark staining chromatin mass within the cells lies either at the end or along the side walls. Many cells are surrounded by a clear capsular-like area.

The vegetative form of the fungus develops when these cells are subcultured on solid media and incubated at room temperature. Growth occurs more rapidly than in the initial isolation and may be visible within five to six days as small, white, downy colonies. This type of growth is characterized by the formation of large, tuberculate spores which are an aid in identifying this and related species and may be seen in wet mounts of the colonies. They have been likened in appearance by DeMoubrenn<sup>3</sup> "to the ancient Tentonic war clubs."

No previous report of the reproduction of systemic disease in guinea pigs has been noted. We have successfully infected this animal by intraperitoneal injection of the yeastlike form. A slowly progressive infection was produced, and at autopsy the typical pathology of histoplasmosis was observed.

Thus far, our attempts to reproduce the disease in mice and rabbits have not been successful.

It is interesting to note that the parasite was seen at autopsy in the kidneys of both the patient and the experimentally infected animal. This offers the future possibility of isolating the organism from the urine. Preliminary experiments with guinea pigs, which will be reported later, bear out this suggestion. We have been able to isolate the organism from this source on several occasions.

Frequently the lesions found at autopsy have been compared with the tubercle. Though the foci vary to a certain extent in various organs according to the stage of development, they differ essentially from the tuberculous lesion in that true epithelioid cells and Langhans' giant cells are absent. The large mononuclear cell reaction in some areas may closely resemble epithelioid cells of an early tubercle, but parallel arrangement in radiating fashion, elongation to spindle-shaped cells, and a resemblance to endothelial or connective tissue elements could nowhere be demonstrated. Furthermore, the lesions of the lung differ essentially from the formation of tubercles in that the tissue undergoes necrosis apparently before proliferative reaction has commenced. The areas of necrosis in contradistinction to tuberculosis show the shadowy outline of the underlying structure and the intact vessels traversing the foci.

The involvement of vessels is a conspicuous feature, particularly the periendothelitis, though this process is not in itself pathognomonic. It has been suggested by Phelps and Mallory,<sup>5</sup> and later by Dodd and Tompkins,<sup>7</sup> that the packing of blood capillaries and sinusoids with leucocytes causes the focal necrosis by ischemia. It is our impression that some toxic process liberated by the organism is responsible for the necrosis, since small vessels and capillaries are abundant and patent, both surrounding the nodular granuloma and even passing through the necrotic areas.

The appearance of the healing stage of the nodular granulomas found in the lung has not yet been described satisfactorily. It is uncertain in our mind

whether the nodules in our case resembling old healed tubercles should be interpreted as healing *Histoplasma capsulatum* granulomas or true tubercles. Finally, it should be mentioned that marked polymorphonuclear cell reaction with abscess formation occurs. This type of cellular reaction has not been described in any other previous case.

#### SUMMARY

A case of systemic histoplasmosis is reported in which diagnosis was made before death by means of smear and culture of the organism from the blood.

Experimental production of the disease in guinea pigs is described.

A detailed description of the pathologic morphology and its differential diagnosis is given.

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# THE PATHOLOGY OF ATROPHIC ARTHRITIS\*

## A CORRELATED CLINICAL AND LABORATORY STUDY

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THE soft and hard tissue changes which take place in rheumatoid or atrophic arthritis proceed in a certain sequence. The swelling, the increase in local heat, and redness of the soft tissues of the joint structure which the clinician observes, and the pain and stiffness of the joints which the patient notices are caused by an active inflammation of the synovial membrane, the capsule, and the surrounding soft tissue structures of the joint. The synovial membrane or stratum synoviale is normally made up of a thin layer of cells richly supplied with nuclei and a subjacent area of connective tissue cells poorly supplied with nuclei. The synovial membrane is richly supplied by a close network of blood vessels and nerves. Fig. 1 is a photomicrograph through normal synovial membrane. In atrophic arthritis there is marked proliferation of the cells of the synovial membrane which eventually produces a layer of granulation tissue or pannus over the articular cartilage. A similar process occurs simultaneously, involving the connective tissue elements of the marrow below the articular cartilage. The latter process extends through the zone of provisional calcification and destroys the articular cartilage from below, while a similar pathologic process evidenced by the proliferated synovial membrane destroys the articular cartilaginous surface. These two opposing processes eventually destroy the articular cartilage. These two layers of active granulation tissue have marked potentialities of forming fibrous and bony tissues, and when they do we have a resulting fibrous or bony ankylosis.

The synovial fluid is increased in the earlier stages and decreased in amount or completely absent in the later stages. Round cell infiltration occurs into the capsule, new blood vessels appear, and fibroblastic activity occurs. Decalcification occurs in the juxtaarticular position. This leads to rarefaction and loss of density. The pathologic picture given so far is essentially in agreement with that given by Nichols and Richardson,<sup>1</sup> Allison and Ghormley,<sup>2</sup> and others.<sup>3</sup> Allison and Ghormley<sup>2</sup> believe that atrophic arthritis has a specific micro-pathology. "Histologically the tissue shows a definite picture, it is as clear-cut as is that of tuberculosis, mainly a proliferative change in the synovial membrane and marrow which is characterized by focal collection of lymphocytes. This microscopic picture will, we believe, be enough to establish the diagnosis of proliferative arthritis, and in our own experience has been enough to predict the subsequent changes in several instances where the tissues showed the character-

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istic changes in the early stages of the clinical disturbances." They emphasize that the focal collections of lymphocytes are not perivascular. Miller<sup>4</sup> adds to this interpretation. He believes that the focal collection of lymphocytes indicates that atrophic arthritis is an inflammatory disease of bacterial origin.

In 1929 Fisher<sup>5</sup> described a histologic picture in atrophic arthritis in which there was perivascular round cell infiltration. Parker and Keefer<sup>6</sup> have recently written an excellent article on the pathologic changes in three cases of atrophic arthritis in which they described the microscopic picture in some regions as showing collections of round cells which were perivascular, as described by Fisher<sup>5</sup> in 1929, and in other regions in which the focal collection of lymphocytes was not perivascular, as described by Allison and Ghormley<sup>2</sup> in 1931. These authors failed to give clinical histories in two of these cases. Such a correlated study would have added to the value of their report. Boyd<sup>7</sup> states that the arteries in the surrounding tissue of the diseased process in atrophic arthritis often show an obliterating arteritis and deposits of fibrin under the endothelium. This vascular change in atrophic arthritis is not mentioned in the other articles previously cited.

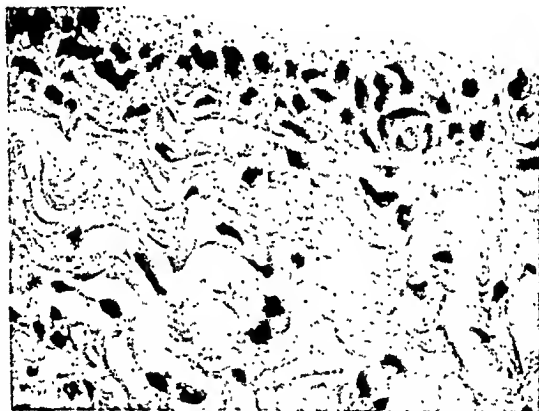


Fig. 1.—A high-power photomicrograph through normal synovial membrane. Note the lining layer of epithelial-like cells and the adjacent layer of connective tissue.

The philosopher Friedrich Nietzsche, who lived in the latter part of the nineteenth century, in his apophthegms and interludes stated, "Everything absolute belongs to pathology." And so it is. In a protean disease, such as atrophic arthritis, which is characterized clinically by remissions and exacerbations, whose onset may be acute or may be quite gradual; which may attack the young, the middle aged, and the aged; which may deform in a very short time, or else may continue in a mild course for many years, one should find different gross and microscopic pathologic findings depending upon the time in which the disease was studied, perhaps the grade of the disease, and the duration of the disease in that particular individual. I have taken case histories of various grades and durations of atrophic arthritis and correlated these with the microscopic pathologic picture to be found in that particular case. It is hoped from this unbiased report that the controversial issues of the pathologic picture of atrophic arthritis may be clarified to some extent.

## CASE REPORTS

CASE 1.—L. G., a white female, aged 60 years, was first seen on April 8, 1935. She complained of painful, swollen stiff joints. She had not been able to get out of her bed for a period of several months due to crippling because of this condition. The onset of her joint trouble began in 1902, with pain and swelling of the proximal interphalangeal joints of both hands. The condition had slowly progressed until it involved practically all the joints. There was a history of two severe flare-ups, one in 1923 and one in 1933. During



Fig. 2A.—A low-power photomicrograph showing a villous mass made up of proliferative synovial tissue in advanced atrophic arthritis.

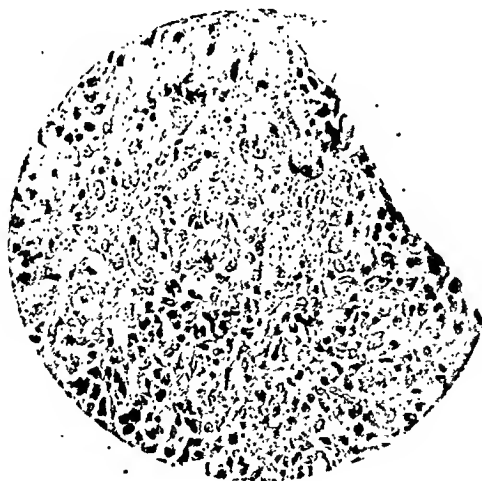


Fig. 2B.—A high-power photomicrograph of the same case showing the marked increase in fibroblasts characteristic of the "healed" or "burnt out" case of atrophic arthritis.

this period of thirty-one years most joints of the body had become involved with pain, swelling, and redness. The examination of the joints on April 8, 1935, revealed little motion in the finger joints, soft tissue swelling of the metacarpophalangeal joints with wasting of the interossei group of muscles, marked limitation of pronation and supination in the elbow olecranon bursa. Both the knee joints were swollen, and there was marked increase thickness of the capsule. Thirty degrees flexion deformity was present in both knees. The ankles were also swollen and quite tender. Blood pressure was systolic 120 and diastolic 80. Lab.

oratory examination revealed a sedimentation rate of 1.00 and a negative Wassermann test. The diagnosis was advanced atrophic arthritis. The patient was treated with bed rest, moist heat to the joints, massage, high caloric diet, and hemolytic streptococcal vaccine. The arthritic process quieted down after two months' treatment. A right synovectomy was done on June 14, 1935. There was marked increase in fluid in the joint, extensive arborescent villous growth, and marked destruction of the cartilage. Microscopic sections of the synovial membrane removed from this joint are shown in Figs. 2A and 2B. One is a low-power

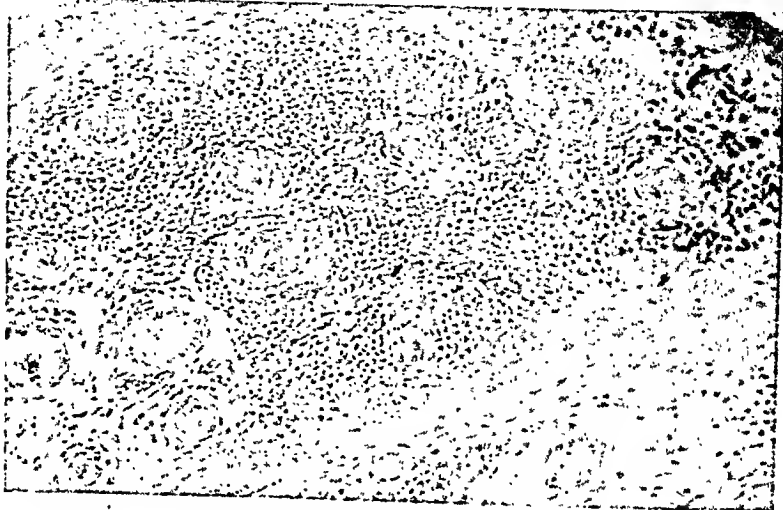


Fig. 3A.—A low-power photomicrograph showing a focal collection of lymphocytes, increase in capillaries, and in fibroblasts.

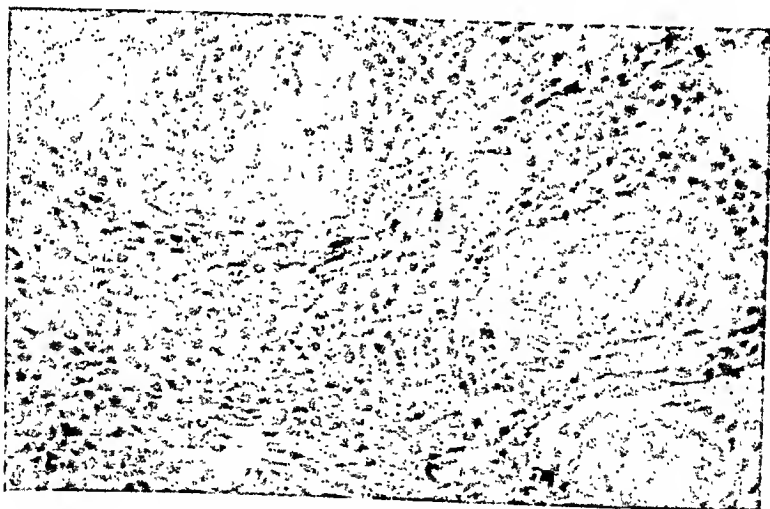


Fig. 3B.—A high-power photomicrograph of the same case showing a diffuse round cell rather than a perivascular round cell infiltration.

and the other is a high-power photomicrograph of this tissue. These sections show marked increase in granulation tissue and areas of hyalinization. There is also diffuse marked increase in round cell infiltration. The high-power photomicrograph particularly shows the increase in fibroblastic activity and the decrease in cellular infiltration. Cultures from the joint and guinea pig inoculation were negative. The pathologic picture in this case was typical of a chronic advanced atrophic arthritis. The marked proliferation of synovial tissue, as evidenced by the villous growth and the replacement of the synovial tissue by fibroblasts and the relative absence of lymphocytes in the synovial membrane in this case, represents the so-called "healed" type or "burnt out" case.

CASE 2.—C. N. H., a white female, aged 47 years, was first seen in February, 1938, complaining of pain and swelling and limitation of motion of the right knee of one month duration. The blood pressure was systolic 120 and diastolic 85. Examination of the right knee revealed diffuse swelling and moderate tenderness of the joint. There was little limitation in motion of this joint. A synovectomy was done, and section through the synovial membrane revealed the so-called typical focal collection of lymphocytes, as described by Allison and Ghormley,<sup>2</sup> increase in fibroblastic activity, and increase in the number of capillaries (Fig. 3A). Culture from the joint and guinea pig inoculation were negative. The high-power photomicrograph of this tissue definitely reveals the absence of perivascular round cell infiltration (Fig. 3B).

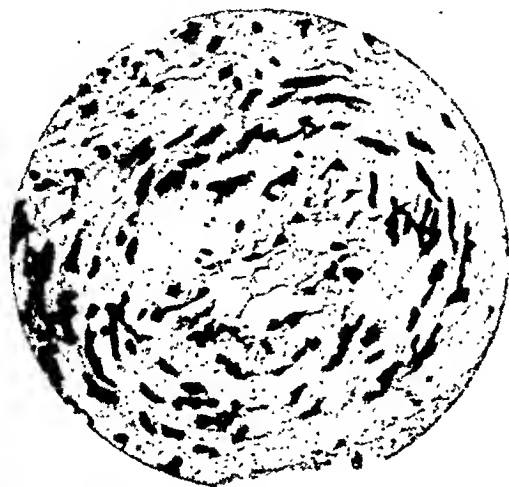


FIG. 4.—A photomicrograph through a blood vessel of the synovial membrane in a case of atrophic and hypertrophic arthritis complicated by essential hypertension and syphilis.

CASE 3.—H. B., a white female, 54 years of age was first seen on July, 1938, complaining of a painful swollen right knee of ten months' duration. Examination of the joint revealed moderate swelling of both joints. An x-ray examination revealed evidence of hypertrophic arthritis as evidenced by new bone production. The blood pressure was systolic 240 and diastolic 118. The patient was a known syphilitic who had been treated for syphilis for five continuous years up until one year before the present observation. Her last Wassermann test was minus, 3 plus, 3 plus. A section through a blood vessel of the synovial membrane of the right knee is shown in photomicrograph Fig. 4. The marked increase in thickness of the muscular wall may be due to the hypertension. The fibrinous exudate in the lumen of the vessel may be due to the syphilitic process. This case emphasizes the need of correlated clinical and pathologic study before conclusions as to the exact pathology of atrophic arthritis are made.

CASE 4.—S. M., a white female, aged 27 years, gave a history of one year's duration of painful swollen joints. The patient first noticed pain and swelling in the smaller articulations, but this gradually progressed until most joints of the body had become involved. She had noticed definite flare-ups associated with upper respiratory infections. The physical examination revealed a well-developed, but poorly nourished, white female. Her tonsils had been removed, and the dental examination both on physical and by radiographic examinations were negative. The posterior pharynx appeared moderately injected. Vaginal examination revealed the patient to be a virgin. Examination of the joints revealed the following: Marked limitation of motion of the right wrist and finger joints; a swollen, inflamed, and quite tender, right knee. Laboratory examination: Sedimentation rate on November 30, 1936, 1.10; serum calcium 11.3; serum phosphorus 4.5; Wassermann reaction negative. Throat

culture revealed both hemolytic streptococci and *Streptococcus viridans*. Vaginal smear and culture were negative. The patient was treated with bed rest, high caloric diet, moist heat, and hemolytic streptococcal vaccine. The sedimentation rate on December 21, 1936, was 0.88. Laboratory examination on January 4, 1937, revealed a sedimentation rate of 0.91, a negative complement fixation test for the gonococcus, a basal metabolism minus 1 per cent; throat culture was negative for hemolytic streptococci. X-ray examination of the joints revealed a negative examination as regards bone structure of the left knee. There was slight narrowing of the joint spaces of the temporomandibular joints. X-ray examination of the right knee on September 16, 1937, was no longer normal. There was roughening of the spine of the tibia, roughening of the joint surface, particularly the lateral aspect.

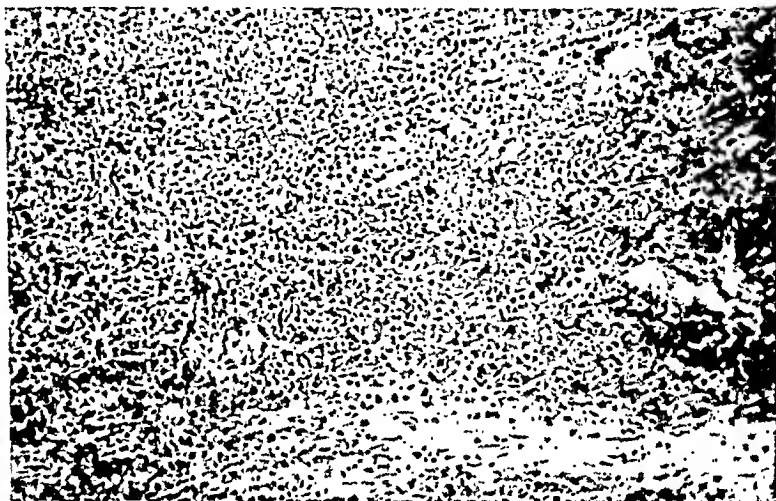


Fig. 5A.—A low-power photomicrograph through the synovial membrane of case S.M. showing both polymorphonuclear and round cell infiltration.

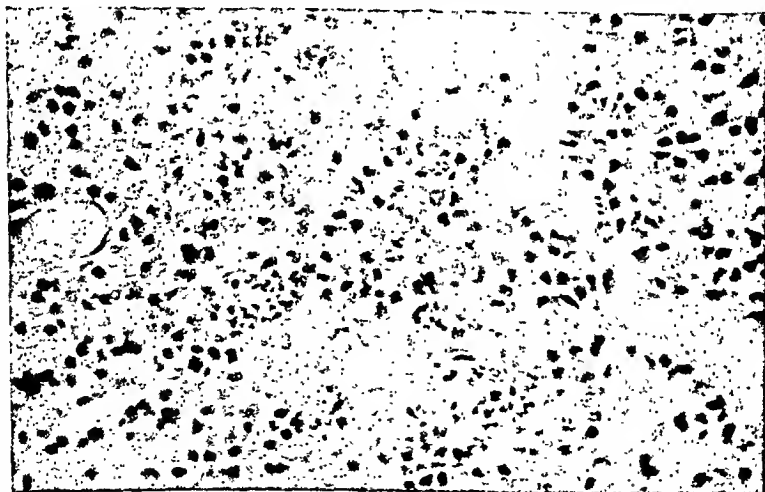


Fig. 5B.—A high-power photomicrograph through the same tissue showing the "purulent" inflammatory reaction characterized by the polymorphonuclear infiltration.

There was also questionable narrowing of the joint space. Both knees and the left ankle were markedly swollen, and there was definite increase in local temperature on January 13, 1938. The sedimentation rate at this examination was 1.53. The patient was treated at home by bed rest, hot baths, general massage, high caloric diet, and hemolytic streptococcal vaccine until November 14, 1938. She had shown marked clinical improvement during this

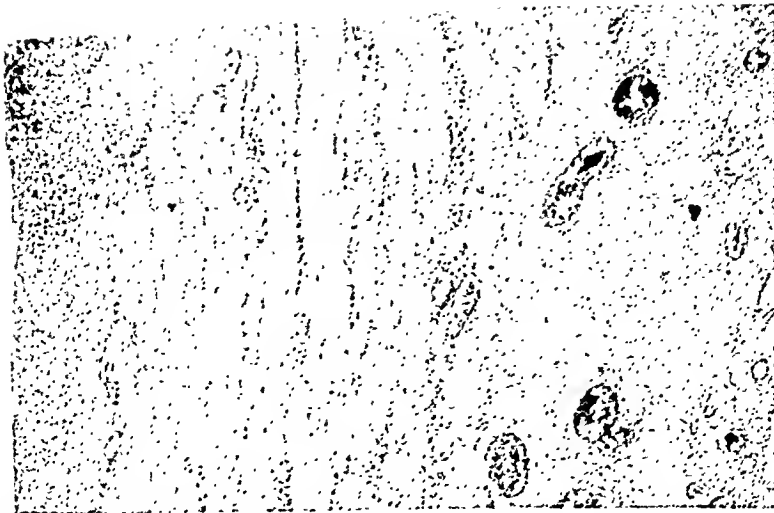


Fig. 6A.—A very low-power photomicrograph through the entire synovial membrane removed from the left knee of case S.M. This shows diffuse round cell infiltration of the entire layer, most pronounced near the surface.

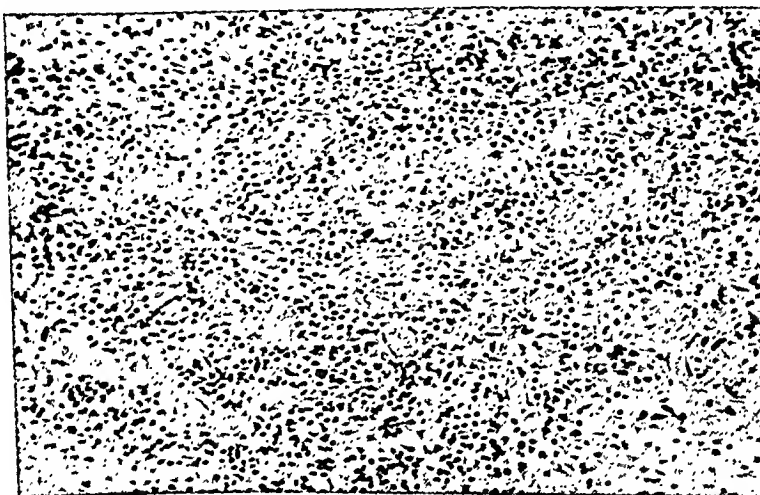


Fig. 6B.—A high-power photomicrograph through the surface layer of case S.M. showing marked round cell infiltration.

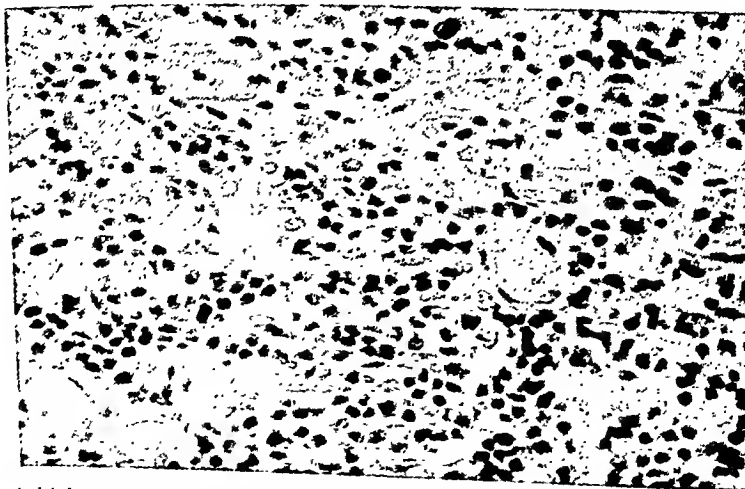


Fig. 6C.—A high-power photomicrograph through the synovial membrane of case S.M. showing diffuse round cell infiltration and increased fibroblastic activity.

period, and it was deemed advisable to do a bilateral synovectomy. A right synovectomy was done on November 21, 1938. Figs. 5 and 5*A* are sections through the synovial membrane removed from the right knee during this operation. Fig. 5*A* is a low-power photomicrograph, and Fig. 5*B* is a high-power photomicrograph. The most striking feature is the presence of polymorphonuclear cells throughout the membrane. In fact, the synovial membrane is much fibrosed, and the inner layer showed a purulent inflammatory reaction, a finding much more common in typical pyogenic arthritis than in atrophic arthritis. The patient made an uneventful recovery, and a synovectomy was done on the left knee on December 14, 1938. Figs. 6*A*, 6*B*, 6*C*, and 6*D* are sections through the synovial membrane removed from this knee joint. Fig. 6*A* is a very low-power photomicrograph, and Figs. 6*B* and 6*C* are high-power photomicrographs. The inner surface of this synovial membrane showed much fibrosis and very marked infiltration of the round cells. This round cell infiltration was diffuse. Fig. 6*C* is a section through the synovial membrane removed from the left knee; it shows definitely that the round cell infiltration is not perivascular. Fig. 6*D*, a very high-power photomicrograph of another section through the same synovial layer, showed a blood vessel with definite perivascular round cell infiltration.

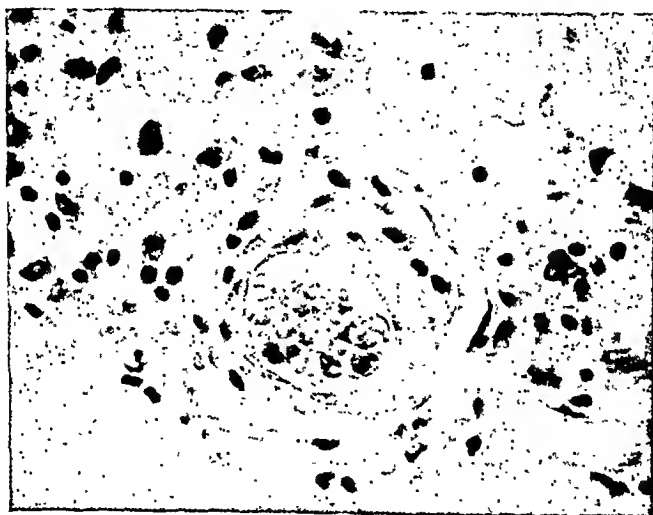


Fig. 6*D*.—A very high-power photomicrograph taken through another area of the synovial membrane in case S.M. showing perivascular round cell infiltration.

#### COMMENT

No specific micropathology exists in chronic atrophic arthritis. The disease is characterized by remissions and exacerbations. It is ushered in rather acutely in some cases and there is marked destruction of the joints in a comparatively short period of time, while in others the disease is more or less quiescent for a period of years. I have seen some cases in which chronic atrophic arthritis has been present for thirty years with no resultant deformities in the joints. I have seen others in which the patient has been made a cripple in the relatively short space of six to eight months. Perhaps it is this protean nature of the disease that accounts for the different pathologic pictures. It is hoped that the photomicrographs shown in this article demonstrate most of the changes that occur in the synovial layer in atrophic arthritis. The picture varies from one in which there is polymorphonuclear infiltration (this section being obtained in a joint which was quite active at the time of operation) to



sections showing round cell infiltration, both perivascular and nonperivascular. A "burnt out" case showing the process after many years of involvement, with little round cell infiltration but replacement of the synovial tissue by means of scar tissue, is shown. It must also be remembered that atrophic arthritis is both a common and a chronic disease, and that, therefore, other diseases may be associated with this condition and may tend to give a varied pathologic picture. Conditions such as syphilis and hypertension, and other diseased conditions, which may affect the blood vessels of the body, must be remembered, since they must be ruled out before concluding that the pathologic picture found in the synovial tissue is the one that is characteristic of atrophic arthritis. It is hoped that correlated clinical and pathologic studies, even though they be a few cases, may offer much in the elucidation of the processes involved in atrophic arthritis.

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## SODIUM SULFAPYRIDINE MONOHYDRATE INTRAVENOUSLY IN THE TREATMENT OF LOBAR PNEUMONIA\*

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CHEMOTHERAPY in the treatment of pneumonia has undergone considerable change in recent years. As a result of the introduction of sulfapyridine in the treatment of the disease, the mortality rate has markedly declined. In 1939 we reported the results of treatment of 100 consecutive cases of lobar pneumonia by oral administration of sulfapyridine. Our mortality rate in that group was 3 per cent. We observed no serious reactions to the drug, although there were some mild toxic manifestations. Occasionally the condition of the pneumonia patient is such that the oral use of this essential therapeutic agent is impossible because of nausea, vomiting, delirium, and other complications. Here the parenteral use of the drug becomes necessary. On the Medical Service of the Edward J. Meyer Memorial Hospital, sodium sulfapyridine monohydrate

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was used intravenously in 25 cases in order to compare its effect when thus given to the oral administration of sulfapyridine.

In this group of cases there were about an equal number of males and females, between the ages of 20 and 70 years, the majority of them being under 50 years of age.

Table I indicates the duration of illness prior to admission to the hospital when treatment was instituted.

TABLE I  
DURATION OF ILLNESS PRIOR TO HOSPITALIZATION

| NO. OF DAYS   | NO. OF CASES | PER CENT |
|---------------|--------------|----------|
| 0-2           | 17           | 68       |
| 3-5           | 5            | 20       |
| 6-9           | 2            | 8        |
| 10-14         | 0            | 0        |
| Above 2 weeks | 1            | 4        |

It will be noted that about one-third of the cases did not enter the hospital until the third day or more after the onset of the disease.

Sputum typing and blood cultures were done on each case, and results are recorded in Table II.

TABLE II  
LABORATORY STUDIES

| TYPE OF PNEUMOCOCCUS | NO. OF CASES | BLOOD CULTURE      | RESULTS               |
|----------------------|--------------|--------------------|-----------------------|
| I                    | 1            | Positive           | Recovered             |
| II                   | 6            | Positive in 1 case | 5 recovered<br>1 died |
| III                  | 2            | Negative           | Recovered             |
| IV                   | 3            | Positive in 1 case | Recovered             |
| VIII                 | 2            | Negative           | Recovered             |
| Undetermined         | 2            | Negative           | Recovered             |
| Negative             | 9            | Negative           | 8 recovered<br>1 died |

This year there appeared to be quite a diversity in the pneumococci types of pneumonia. Usually we find a more predominant type, but the virulence in each particular type varies considerably from time to time. Blood counts were done in each case daily. The majority of our cases had a leucocytosis prior to the administration of the drug. In only rare instances did we use the drug when the white blood count was less than 10,000.

Table III shows the blood counts before treatment.

TABLE III  
BLOOD COUNTS

| W.B.C.    | NO. OF CASES | POLYS. | NO. OF CASES |
|-----------|--------------|--------|--------------|
| 0- 5,000  | 0            | 50- 60 | 1            |
| 5-10,000  | 3            | 60- 70 | 5            |
| 10-20,000 | 17           | 70- 80 | 6            |
| 20-30,000 | 4            | 80- 90 | 10           |
| 30-40,000 | 1            | 90-100 | 3            |

TABLE IV  
GENERAL ANALYSIS OF BLOOD CONCENTRATION

| CASE | DRUG<br>STARTED | FIRST INJECTION<br>(MG. % IN BLOOD) |       |       |       |                | SECOND INJECTION<br>(MG. % IN BLOOD) |       |       |       |                | THIRD INJECTION<br>(MG. %) |       |       | FOURTH<br>INJEC-<br>TION | DRUG<br>STOPPED | TOTAL<br>MG.<br>DRUG<br>USED | TEMP.<br>ON<br>ADM. | DAY OF<br>CHISIS<br>OR<br>LYSIS |
|------|-----------------|-------------------------------------|-------|-------|-------|----------------|--------------------------------------|-------|-------|-------|----------------|----------------------------|-------|-------|--------------------------|-----------------|------------------------------|---------------------|---------------------------------|
|      |                 | 10<br>MIN.                          | 1 HR. | 4 HR. | 8 HR. | 24 HR.         | 10<br>MIN.                           | 1 HR. | 4 HR. | 8 HR. | 24 HR.         | 10<br>MIN.                 | 1 HR. | 4 HR. |                          |                 |                              |                     |                                 |
| H.D. | 4/27/40         | 7.7                                 | 5.9   | 4.2   | -     | Less<br>than 1 | 7.6                                  | 5.5   | 4.2   | -     | Less<br>than 1 | 3.7                        | 1.9   | 1     | -                        | 5/ 1/40         | 20                           | 105                 | 4th                             |
| L.P. | 4/28/40         | 8.7                                 | 8     | 7.1   | 5.3   | 4.3            | -                                    | -     | -     | 8.9   | 3.8            | -                          | -     | -     | -                        | 5/ 1/40         | 12                           | 105                 | 4th                             |
| C.S. | 4/30/40         | 8.9                                 | 7.6   | 2.8   | 2     | 0.7            | 8.8                                  | 7.3   | 2     | 3.8   | 1              | -                          | -     | -     | -                        | 5/ 2/40         | 12                           | 101.3               | 2nd                             |
| F.R. | 5/ 6/40         | 7                                   | 6     | 5     | 4.5   | 6              | 12                                   | 10    | 11    | 10    | 2              | 10                         | -     | -     | -                        | 5/11/40         | 20                           | 100.6               | 2nd                             |
| D.S. | 5/14/40         | 11                                  | 7     | 6     | 6     | 6              | 10                                   | 10    | 10    | 10    | 6              | 10                         | -     | -     | -                        | 5/18/40         | 20                           | 101.8               | 2nd                             |
| J.S. | 5/14/40         | 10                                  | 10    | 7     | 6     | 7              | 12                                   | 12    | 10    | 10    | 5              | 4                          | -     | -     | -                        | 5/18/40         | 20                           | 102.3               | 3rd                             |
| L.K. | 5/15/40         | 8                                   | 7     | 5     | 1     | 1              | 5                                    | 5     | 5     | Trace | -              | -                          | -     | -     | -                        | 5/22/40         | 28                           | 102.0               | 6th                             |
| C.H. | 5/17/40         | 9                                   | 6     | 7     | 6     | 3              | 5                                    | 4     | 7     | 6     | 4              | -                          | -     | -     | -                        | 5/22/40         | 24                           | 103.2               | 3rd                             |
| C.S. | 5/23/40         | 9                                   | 5     | 5     | 4     | 1              | 10                                   | 8     | 7     | 6     | 4              | -                          | -     | -     | -                        | 5/26/40         | 16                           | 103.6               | 3rd                             |
| L.T. | 8/23/40         | 11                                  | 9     | 8.4   | 6.6   | 4.1            | 17.9                                 | 14.8  | 12.6  | 10    | 5.6            | -                          | -     | -     | -                        | 8/27/40         | 20                           | 102.8               | 2nd                             |
| H.P. | 7/27/40         | 4                                   | 4     | -     | 3.4   | 3              | 2.2                                  | 7.4   | 5.8   | 4     | 3.5            | 2.3                        | -     | -     | -                        | 8/ 1/40         | 20                           | 105.4               | 8th                             |
| M.S. | 7/27/40         | 5.9                                 | 7.5   | 8.6   | 11    | 9.1            | 10.1                                 | 9     | 8.3   | 4     | 1              | -                          | -     | -     | 10.8                     | 7/31/40         | 20                           | 102.6               | 3rd                             |
| N.S. | 7/27/40         | 4.8                                 | 4     | 3.2   | 3     | 2              | 6                                    | 5.4   | 4     | 4     | 3              | -                          | -     | -     | 8                        | 7/30/40         | 12                           | 105                 | 2nd                             |
| S.H. | 6/ 2/40         | 2                                   | 8     | 5     | 4     | 5              | 14                                   | -     | 9     | 8     | -              | -                          | -     | -     | -                        | 6/4 /40         | 10                           | 102.8               | 4th                             |
| M.D. | 5/28/40         | 7                                   | 5     | 7     | 8     | 1              | 8                                    | 6     | 5     | 4     | Trace          | -                          | -     | -     | 7                        | 5/31/40         | 16                           | 100.2               | 5th                             |
| S.P. | 5/27/40         | 7                                   | 5     | 5     | 1     | 1              | 5.6                                  | 4.8   | 5     | 4     | -              | -                          | -     | -     | -                        | 5/31/40         | 20                           | 104.8               | 8th                             |
| N.H. | 5/29/40         | 5.2                                 | 3.9   | 3.8   | 3.4   | -              | 14                                   | 6     | 9     | 4     | Trace          | -                          | -     | -     | -                        | 5/31/40         | 12                           | 102.6               | -                               |
| H.D. | 10/ 6/40        | 10.9                                | 9.1   | 13.1  | 6     | 11.4           | 9.1                                  | 11.3  | 10    | 9.4   | 9              | -                          | -     | -     | -                        | 10/13/40        | 24                           | 101.8               | 2nd                             |
| B.H. | 10/15/40        | 20                                  | 13.3  | 10    | 8.3   | 7              | 18                                   | 14    | 9     | -     | -              | -                          | -     | -     | -                        | 10/17/40        | 12                           | 104                 | 2nd                             |
| G.B. | 9/28/40         | 10                                  | 7.8   | 6.9   | 5.6   | 13.8           | 10.3                                 | 8.8   | 7.8   | -     | 29.6           | -                          | -     | -     | -                        | 9/30/40         | 12                           | 99.4                | 2nd                             |
| L.N. | 10/13/40        | 7.8                                 | 5.4   | 5.1   | 4.1   | 8.8            | 10.4                                 | 7.2   | 5.8   | -     | -              | -                          | -     | -     | -                        | 10/14/40        | 8                            | 101                 | 2nd                             |
| J.R. | 10/18/40        | 8                                   | 6.7   | 4.6   | -     | 10.7           | 7.6                                  | 7.0   | 7.6   | -     | -              | -                          | -     | -     | -                        | 10/20/40        | 12                           | 103                 | 2nd                             |
| R.T. | 10/18/40        | 4.2                                 | 3.8   | 3.3   | -     | 5.9            | 6.7                                  | 3.1   | 5.9   | 7.6   | -              | -                          | -     | -     | -                        | 10/21/40        | 16                           | 101                 | 2nd                             |
| H.F. | 10/17/40        | 6.3                                 | 4     | 2     | -     | -              | 3                                    | 4.6   | 8     | 4     | -              | -                          | -     | -     | -                        | 10/21/40        | 20                           | 103                 | 4th                             |
| R.B. | 10/15/40        | 8                                   | 5.3   | 4     | 5     | 4.3            | -                                    | -     | 7     | 4     | -              | -                          | -     | -     | -                        | 10/20/40        | 25                           | 101                 | 3rd                             |

## METHOD OF ADMINISTRATION OF SODIUM SULFAPYRIDINE MONOHYDRATE

After the diagnosis of lobar pneumonia had been made and corroborated by x-ray, each patient was placed on a liquid diet containing a minimum of three liters per day, made up of fruit juices and milk. If oral fluids were not taken in sufficient quantity, we resorted to parenteral administration of saline and glucose. The only other routine therapeutic agent used was an opiate if indicated for cough or sedation, and in some instances oxygen by nasal catheter. The latter, however, did not seem to relieve the cyanosis when present while this drug was being used.

The sodium sulfapyridine was administered slowly intravenously, using a 5 per cent solution (4 Gm. of the powder dissolved in 80 c.c. of distilled water). Eighty cubic centimeters were given daily, and the blood was examined for per cent of sulfapyridine content at frequent intervals during the twenty-four hours. In rare instances the dose was repeated before the twenty-four hours were up in order to maintain a higher blood level. We did not supplement this therapy with the oral use of the drug, since we desired to determine its effect by the intravenous method only.

TABLE V  
REACTIONS

| REACTION          | INTRAVENOUS<br>25 CASES (%) | ORAL<br>100 CASES (%) |
|-------------------|-----------------------------|-----------------------|
| Nausea and emesis | 84                          | 17                    |
| Cyanosis          | 52                          | 18                    |
| Headache          | 36                          | None                  |
| Delirium          | 16                          | 4                     |
| Jaundice          | 12                          | None                  |

By this method of administration when a single dose was given daily, irrespective of size of the patient, the per cent of sulfapyridine in the blood shortly following injection (ten minutes) reached an average level of 8 mg. per cent and dropped about 20 per cent after an interval of one hour. Determinations at the end of four hours showed an additional 5 per cent drop. At the eighth hour there was an average decrease in sulfapyridine of 37.5 per cent. Recording of blood level for the drug at the end of twenty-four hours showed no appreciable change from the eight-hour level, the average being about 5 mg. per cent. Since it is advisable to maintain a blood level between 5 and 10 mg. per cent, it seems that the drug should be administered at least twice daily, and in some instances three times daily. With the above dosage the blood concentration, as noted in Table IV, varies considerably in each patient, irrespective of age, sex, or weight. When using sulfapyridine orally, the dose used was 2 Gm. on admission, followed by 1 Gm. every six hours and continued for three days following drop of temperature to normal. The blood concentration level on the average in twenty-four hours, with use of the oral drug, was 3.93 mg. per cent, while with the intravenous drug given once daily, it was about 5.6 mg. per cent. The duration of illness following the intravenous administration of the drug varied somewhat. In about 40 per cent of the cases the temperature came down by lysis or crisis within forty-eight hours. In one-third of the group this oc-

curred within four days. The remainder varied between five and eight days, none more than eight days. This compared closely with the data obtained with the oral use of the drug.

Reactions were similar to those obtained when sulfapyridine was used orally. Nausea and emesis were present in a large group of our cases with the intravenous use of the drug. Cyanosis was more marked, headache was present in a third of the group, jaundice (hemolytic) was observed in two cases (none in oral group), and a toxic hepatitis with regurgitant jaundice was noted in one case.

#### SUMMARY AND CONCLUSIONS

In this group of 25 cases we have had two deaths: one was a senile patient with hyperthyroidism who died from heart failure; the other had type II pneumonia and developed toxic hepatitis. Of significance in our group was a patient with an eight months' pregnancy who was delivered and recovered. Another had an infected abortion; she developed pneumonia and recovered. One had malignant hypertension and uremia, recovered from the pneumonia, but died some weeks later from his cardiovascular disease. We do not consider his death due to pneumonia.

The intravenous use of sodium sulfapyridine monohydrate has no particular advantage over the oral use of sulfapyridine or sulfathiazole when the latter can be taken orally by the patient. In those patients who for some reason are unable to take the drug orally, the intravenous sodium sulfapyridine should be used in conjunction with pneumonia sera when indicated. From experience, one to two injections daily of 80 c.c. of 5 per cent solution are required to maintain a blood concentration of 5 to 10 mg. per cent. If laboratory facilities are available, one could, by frequent blood examination, determine the exact frequency for the administration of the intravenous drug.

#### REFERENCE

1. Levitt, Abel, Levy, Dexter S., and Jaskiewicz, Stanley J.: Clinical Experience With Sulfapyridine in the Treatment of Lobar Pneumonia, *New York State J. Med.* 39: 1916, 1939.

333 LINWOOD AVENUE

## THE AVERAGE LENGTH OF LIFE OF THE RED CORPUSCLE<sup>o</sup>

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IN THIS study an attempt is made to add to the knowledge of the ever recurrent problem of the length of life of the red corpuscle. Hunter (1884 and 1887), and later Ashby (1919), have given a very good brief history of early investigations. Ashby (1919) also explains why, as yet, we cannot consider the problem solved. Escobar and Baldwin (1934) add somewhat to these early investigations.

Many chemists because they have perfected accurate methods for bile pigment estimation feel that they can thus determine the number of corpuscles destroyed every day and hence the number made every day if their number remains constant. Whipple (1926) criticizes this view by pointing out that there are at least two unknown factors which could seriously disturb these calculations. First, muscle hemoglobin certainly contributes to the amount of bile pigment. Second, hemoglobin set free in the circulation may be changed to bile pigment and urobilin, or it may be used again in hemoglobin construction. Miss Ashby (1919) (1921)<sup>1, 2</sup> has done the most acceptable work so far on this subject. She has shown that the transfused corpuscles live in a recipient's blood stream for thirty days or more. The chief objections to accepting her results as far as the length of life of the corpuscles is concerned have been discussed by the others. Wean and Warren<sup>17</sup> point out that what may be the length of life of a transfused corpuscle may not be the length of life of a native corpuscle. Foreign cells, especially of a different blood group, which necessarily were used by Miss Ashby, may not be as susceptible as native cells to ordinary means of destruction. A second objection is made by Rous (1923) that the "foreign" protein of the stroma of the transfused corpuscles may be utilized over and over again by the recipient and thus form corpuscles unlike the usual corpuscles of the recipient.

Eaton and Danren (1930)<sup>3, 6</sup> explained a method for the estimation of the average length of life of the red corpuscle which has been verified only recently by kinsmen Moore and Harrison (1940). They considered the red blood corpuscles of the blood stream as a population. In a population that remains constant, as many individuals die as are born, or vice versa. If in that population a catastrophe occurs which destroys one-third or one-fourth of the population, immediately an increased number of blood corpuscles, all approximately the same age, is thrown into the blood stream. These new individuals

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that restore the population are called reticulocytes and can be followed by using a special technique (Spohr, 1930). The new thing that Eaton and Damren (1930)<sup>5, 6</sup> point out is the fact that in a population where there are so many individuals of the same age, these individuals finally arrive at that age, corresponding to their average age; then a greater than the usual number die at the same time. This intravascular hemorrhage is sufficient to stimulate another noticeable reticulocyte production. They claim that the average length of life of the corpuscle is the length of time between two successive peaks of reticulocyte production. The one objection to their conclusion is that the second peak of reticulocyte production would occur *at an appropriate distance after the death of a great number of corpuscles that had lived their average length of life and that had appeared in response to the initial hemorrhage.*

By making a daily total erythrocyte count along with the daily reticulocyte count, any unusual population of old corpuscles dying at one time will show itself in the drop in erythrocyte count. This "death drop" may amount to as much as a million per cubic millimeter of blood within a period of two days, as will be shown in the subsequent description of my experiments upon the rabbit. That this sudden death of erythrocytes represents the termination of the natural length of life of the reticulocytes born in response to the previous hemorrhage can scarcely be doubted.

*Definition of Reticulocytes.*—Any mammalian red corpuscle showing a stippling, tiny threads, or network light or heavy when stained with brilliant cresyl blue is called, in this paper, a reticulocyte. Key (1921) explains this basophilic substance in a rather complete manner. King (1928)<sup>12</sup> also gives a concise statement concerning the reticulocyte and Cunningham (1920) describes them rather well.

*Experimental Procedure.*—Well-nourished rabbits, as determined by weight and red blood count of between six and seven millions and a high hemoglobin percentage, and of known age were kept under uniform conditions in the laboratory. The food consisted of mixed ground grain in pellet form, alfalfa hay, and crushed oats with some green food every day.

A control period of several days was run in the case of each rabbit to determine the normal variation in the percentage of reticulocytes, red corpuscles per cubic millimeter of blood, and in one case the hemoglobin per cent. If an animal had more than 2.5 per cent, that animal was discarded. The drop of blood for the daily test was withdrawn from the marginal ear vein by the puncture method, at approximately the same time each day and before the animal was fed.

The blood for the hemorrhage was removed directly from the heart by means of a hypodermic needle and without the use of an anesthetic. An amount of blood estimated to be one-fourth to one-third of the total blood, or about 1.25 per cent of the body weight, was withdrawn. The blood was withdrawn slowly so that the vasomotor system could compensate for its loss. The animal was held quietly by the assistant for a period of five or ten minutes after the hemorrhage and then put back in his hutch and fed and watered as usual.

A period of forty-eight hours after hemorrhage was allowed to pass before the daily tests were begun again. Male rabbits only were used.

*Staining Technique.*—I am indebted to Dr. J. A. Badertscher for the method used in staining the reticulocytes of mammalian blood. The method is simple, quick, and accurate and has not been published.

A drop of fresh blood is placed directly on a glass microscope slide, taken from the ear of a rabbit or finger of a human being. A glass rod, on the end of which is deposited brilliant cresyl blue stain,<sup>\*</sup> is used to stir the blood until it is colored a uniformly greenish blue. The edge of another microscope slide is then placed in the droplet of blue-colored blood. Sufficient time is allowed for the blood to flow along the edge of the touching surface of this latter slide. Then the blood on the edge of this latter or second glass slide is spread on to a third slide by pushing it along on the surface of the third slide in the usual way of making a blood smear. As many as eight or ten blood smears can be made on as many different glass slides from the one drop of stained blood, providing the edge of a clean slide is used every time for spreading the blood on other clean slides.

As soon as the blood smear is made and before it is dry, it should be held in a moist chamber a few seconds. The mouth is a very good moist chamber, providing the breath is held while the slide is in it. This permits the corpuscles to be exposed to a saturated stain longer than otherwise, and there is less distortion of the corpuscles. The moisture of the breath, if blown on the slide, will cause hemolysis of the corpuscles. The smear dries in a minute or two after it is removed from the moist chamber and is then ready to be counterstained with Wright's stain, mounted in balsam, and examined at any subsequent time.

*Counting.*—In the determination of reticulocyte percentage, the cells were counted by means of an ocular Micrometer Disc. The total red blood cell count and the reticulocyte cell count for successive squares were each determined. This was continued until a thousand or more cells had been counted and then the percentage of reticulocytes was estimated.

In making the red blood corpuscle count for the total number of red corpuscles per cubic millimeter of blood, Trenner diluting pipettes and Levy-Hausser counting chambers were used.

## RESULTS

*Young Rabbit.*—Table I and Fig. 1 present the results of an experiment on a young rabbit of from six to seven months old. The experiment lasted twenty-nine days. The conditions of the blood were quite regular during the control period of six days. The average number of red blood corpuscles per cubic millimeter of blood remained constant near 6.4 million, and the hemoglobin estimate was 92 per cent. The reticulocyte percentage fluctuated only slightly, which fluctuation could reasonably be within experimental error. The reticulo-

<sup>\*</sup>A saturated solution of brilliant cresyl blue, using 100 per cent alcohol as a solvent, is used. The tip of a glass rod is dipped in the solution, and the alcohol is burned off in the flame of an alcohol lamp. The glass rod is dipped three or four times until sufficient dye is deposited on the glass rod.



eyte percentage averaged 2.4 per cent, which would make the actual number of reticulocytes present at any one time, per cubic millimeter of blood, about 153,600.

On the sixth day of the experiment after the three blood tests mentioned above had been made, the animal was bled of an estimated one-fourth to one-third of its blood, or about 40 c.c. A period of forty-eight hours was allowed to elapse in order that the liquids of the body could adjust themselves, and then the blood was studied again daily until the end of the experiment. On the eighth day of the experiment, two days after the hemorrhage, a marked change due to prehemorrhagic conditions was shown in the blood when the red corpuscle count, reticulocyte percentage, and hemoglobin percentage were taken. The red corpuscle count had fallen to 4.1 million, the hemoglobin percentage to 56, and the percentage of reticulocytes had increased to 5.1.

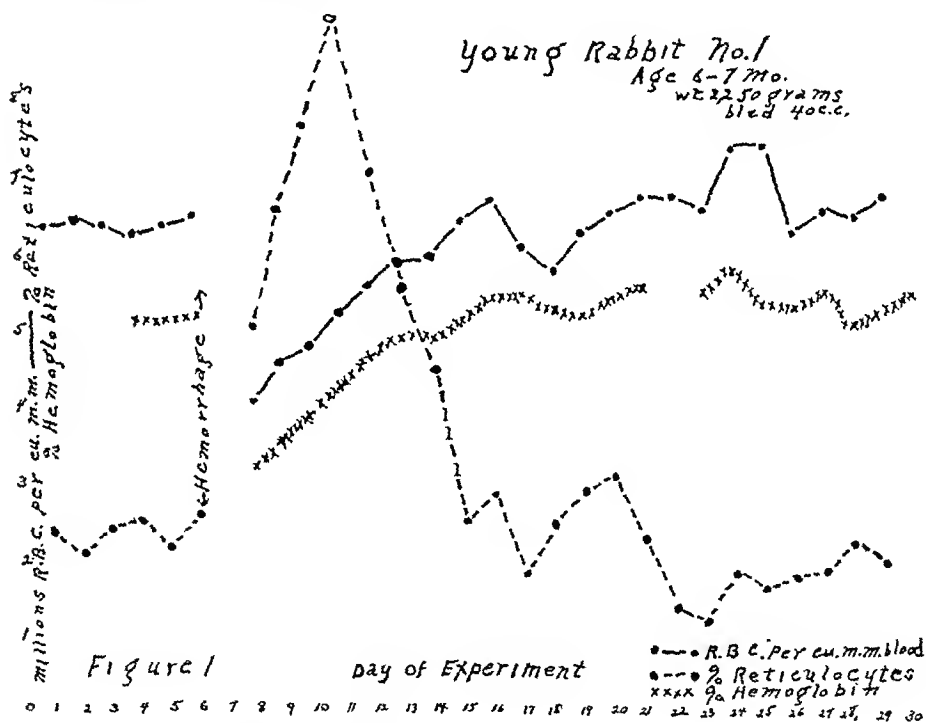


Fig. 1.—The per cent of reticulocytes, the total red corpuscle count, and the hemoglobin percentage were taken before and after a hemorrhage of 40 c.c. of blood, approximately one-fourth of its blood. These were taken on the day of the hemorrhage and again after forty-eight hours. Three peaks of reticulocyte formation occurred on the 11th, 20th, and 28th days of the experiment. The total red corpuscle count was lowest on the 13th and 26th days of the experiment. The average length of life of the red corpuscles can be estimated by counting the days between the 11th and 18th days and between the 20th and 26th days. The average length of life of the red corpuscles was 6.5 days.

The increase in reticulocytes was actual as well as relative. Although the erythrocyte number per cubic millimeter was less than two-thirds the pre-hemorrhagic number, the actual number of reticulocytes per cubic millimeter was 209,100 instead of the 153,600 actually present at any one time in a cubic millimeter of blood during the control period. The expected drop in the number of erythrocytes, if one-third of the blood had been removed, would have been to 4.3 million, and the actual number was approximately 4.0 million. The per-

centage of reticulocytes gradually increased up to 9 per cent on the eleventh day of the experiment, and the whole cycle of reticulocyte formation lasted until the seventeenth day of the experiment. The actual number of reticulocytes present per cubic millimeter of blood at the peak of their production was 450,000 as compared to the 153,600 during the control period. The per cent of hemoglobin and the total number of erythrocytes reached normal levels nine days, sometimes ten, after hemorrhage on the one hundred and forty-fifth or fifteenth day of the experiment, and fluctuated near that level, steadily working upward, however, until the end of the experiment.

TABLE I

YOUNG RABBIT NO. 1 (MALE)

Aged 6-7 Mo. Weight 2,250 Gm. Bled 40 c.c. or One-Third of Blood

| DATE OF EXPERIMENT | DATE  | R.B.C.<br>MILLION PER<br>CU. MM. OF<br>BLOOD | PER CENT<br>HEMOGLOBIN | TOTAL CELLS<br>COUNTED | TOTAL<br>RETICULO-<br>CYTES | PER CENT OF<br>RETICULO-<br>CYTES |
|--------------------|-------|--|------------------------|------------------------|-----------------------------|-----------------------------------|
|                    | 1932  |  |                        |                        |                             |                                   |
| 1                  | 11/14 | 6.4  | ----                   | 1,199                  | 30                          | 2.5                               |
| 2                  | 11/15 | 6.5  | ----                   | 1,302                  | 29                          | 2.2                               |
| 3                  | 11/16 | 6.4  | ----                   | 1,230                  | 32                          | 2.5                               |
| 4                  | 11/17 | 6.3  | 93.0                   | 1,346                  | 36                          | 2.6                               |
| 5                  | 11/18 | 6.4  | 93.5                   | 1,340                  | 29                          | 2.2                               |
| 6                  | 11/19 | 6.5  | 94.4                   | 1,056                  | 30                          | 2.7                               |
| 7                  | 11/20 | ---  | Bled 30 c.c. of blood  | ---                    | ---                         | ---                               |
| 8                  | 11/21 | 4.1  | 56.0                   | 1,037                  | 58                          | 5.1                               |
| 9                  | 11/22 | 4.0  | 62.0                   | 1,209                  | 80                          | 6.6                               |
| 10                 | 11/23 | 4.8  | 69.0                   | 1,122                  | 87                          | 7.6                               |
| 11                 | 11/24 | 5.3  | 76.0                   | 1,063                  | 96                          | (9.0)                             |
| 12                 | 11/25 | 5.6  | 84.0                   | 1,075                  | 75                          | 7.0                               |
| 13                 | 11/26 | 5.9  | 88.0                   | 1,052                  | 59                          | 5.6                               |
| 14                 | 11/27 | 5.9  | 87.0                   | 1,122                  | 50                          | 4.5                               |
| 15                 | 11/28 | 6.4  | 93.0                   | 1,067                  | 28                          | 2.6                               |
| 16                 | 11/29 | 6.6  | 99.0                   | 1,101                  | 32                          | 2.9                               |
| 17                 | 11/30 | 6.0  | 99.0                   | 1,065                  | 20                          | 1.9                               |
| 18                 | 12/ 1 | 5.8  | 95.0                   | 1,195                  | 31                          | 2.6                               |
| 19                 | 12/ 2 | 6.3  | 93.0                   | 1,299                  | 39                          | 3.0                               |
| 20                 | 12/ 3 | 6.5  | 99.0                   | 1,088                  | 37                          | (3.2)                             |
| 21                 | 12/ 4 | 6.7  | 101.0                  | 1,237                  | 29                          | 2.3                               |
| 22                 | 12/ 5 | 6.7  | ----                   | 895                    | 14                          | 1.5                               |
| 23                 | 12/ 6 | 6.5  | 100.0                  | 1,107                  | 15                          | 1.3                               |
| 24                 | 12/ 7 | 7.3  | 105.0                  | 985                    | 19                          | 1.9                               |
| 25                 | 12/ 8 | 7.3  | 96.5                   | 1,486                  | 24                          | 1.7                               |
| 26                 | 12/ 9 | 6.2  | 96.0                   | 1,055                  | 20                          | 1.9                               |
| 27                 | 12/10 | 6.5  | 100.5                  | 1,226                  | 25                          | 2.0                               |
| 28                 | 12/11 | 6.4  | 92.0                   | 1,221                  | 28                          | (2.3)                             |
| 29                 | 12/12 | 6.7  | 94.0                   | 1,027                  | 21                          | 2.0                               |

The rate of reticulocyte production remained higher than during the control period until the number of erythrocytes and the per cent of hemoglobin were almost normal on the fourteenth or fifteenth day of the experiment. On the sixteenth day the erythrocyte number was actually above the control period. The reticulocyte curve continued to fall for two or three more days, fifteenth to seventeenth day. On the eighteenth and nineteenth days there was a rather sudden drop, which amounted to almost a million per cubic millimeter of blood in the number of erythrocytes. The hemoglobin curve followed the erythrocyte

curve, more or less, except that there seemed to be a slight tendency for the hemoglobin curve to lag behind the erythrocyte curve toward the end of the experiment.

The rapid fall in number of erythrocytes on the seventeenth and eighteenth days was equal to a hemorrhage and can well be called an intravascular hemorrhage. It was sufficiently severe to stimulate another wave of reticulocyte production. This peak of reticulocyte production was on the twentieth day. As a result, the erythrocyte number returned to normal and even went quite above normal, reaching a new peak on the twenty-fourth and twenty-fifth days. At the same time the reticulocytes fell below the normal production. The third slight peak of reticulocyte production on the twenty-eighth day can scarcely be regarded as a response to the drop in erythrocytes on the twenty-sixth day, since the total does not go below normal. Because the total red corpuscle count ran above normal, the per cent of reticulocytes was below normal.

TABLE II

OLD RABBIT NO. 2 (MALE)

Aged 5½ Yr. to 6 Yr. Old. Weight 3,300 Gm. Bled 40 c.c. or One-Fourth of Blood

| DATE OF EXPERIMENT | DATE  | R.B.C.<br>MILLION PER<br>CC. MM. OF<br>BLOOD | TOTAL CELLS<br>COUNTED       | TOTAL<br>RETICULOCYTES | PER CENT<br>OF<br>RETICULO-<br>CYTES |
|--------------------|-------|--|------------------------------|------------------------|--------------------------------------|
|                    | 1932  |  |                              |                        |                                      |
| 1                  | 10/ 6 | ---  | 993                          | 16                     | 1.71                                 |
| 2                  | 10/ 7 | 6.3  | 1,054                        | 18                     | 1.70                                 |
| 3                  | 10/ 8 | 6.2  | 1,059                        | 16                     | 1.5                                  |
| 4                  | 10/ 9 | 6.0  | 1,137                        | 19                     | 1.7                                  |
| 5                  | 10/10 | 5.9  | 977                          | 13                     | 1.3+                                 |
| 6                  | 10/11 | 5.8  | 1,510                        | 29                     | 1.9                                  |
| 7                  | 10/12 | 5.9  | 835                          | 10                     | 1.2                                  |
| 8                  | 10/13 | ---  | 1,241                        | 17                     | 1.3+                                 |
| 9                  | 10/14 | ---  | 1,088                        | 21                     | 1.9                                  |
| 10                 | 10/15 | ---  | Bled 35-40 c.c. of blood     |                        | ---                                  |
| 11                 | 10/16 | ---  |                              |                        | ---                                  |
| 12                 | 10/17 | 4.5  | 1,112                        | ---                    | ---                                  |
| 13                 | 10/18 | 4.4  | 1,053                        | 38                     | 3.4                                  |
| 14                 | 10/19 | 4.7  | 1,008                        | 64                     | (6.0)                                |
| 15                 | 10/20 | 4.6  | 1,079                        | 42                     | 4.1                                  |
| 16                 | 10/21 | 4.6  | 1,074                        | 40                     | 3.8                                  |
| 17                 | 10/22 | 3.7  | 949                          | 35                     | 3.2                                  |
| 18                 | 10/23 | 3.4  | 1,123                        | 21                     | 2.2                                  |
| 19                 | 10/24 | 4.0  | 1,339                        | 23                     | 2.0                                  |
| 20                 | 10/25 | 4.2  | 1,110                        | 51                     | 3.8                                  |
| 21                 | 10/26 | 3.9  | 971                          | 52                     | (4.6)                                |
| 22                 | 10/27 | 4.4  | Abnormal erythrocytes appear |                        | 37                                   |
| 23                 | 10/28 | 3.8  |                              |                        | 32                                   |
| 24                 | 10/29 | 3.8  | 1,198                        | 37                     | 3.0                                  |
| 25                 | 10/30 | ---  | 1,046                        | 43                     | 3.0                                  |
|                    |       |  | Died                         | ---                    | 4.1                                  |

The period of time between the greatest production of reticulocytes on the eleventh day and the fall in the number of erythrocytes about the eighteenth day must be regarded as the average length of life of the red corpuscle. This period is approximately seven days. It is also quite possible that the time between the second peak of reticulocyte production at the nineteenth or twentieth days, and the fall in erythrocyte number on the twenty-sixth day, would represent the same thing. Here, too, the time is approximately seven days.

*Old Rabbit.*—An experiment similar to the above was performed on an old rabbit (5½ to 6 years), the details of which are presented in Fig. 2. The experiment covered a period of twenty-four days, at which time the animal died. This rabbit, although old, was in good condition until on or near the twenty-first day, three days before death, the rabbit showed signs of weakness and diarrhea, probably due to too large an amount of green food during the weakened period subsequent to hemorrhage. The reticulocytes stained more deeply at this time, and can be considered polychromatophiles. The important part of the experiment was completed before these symptoms appeared.

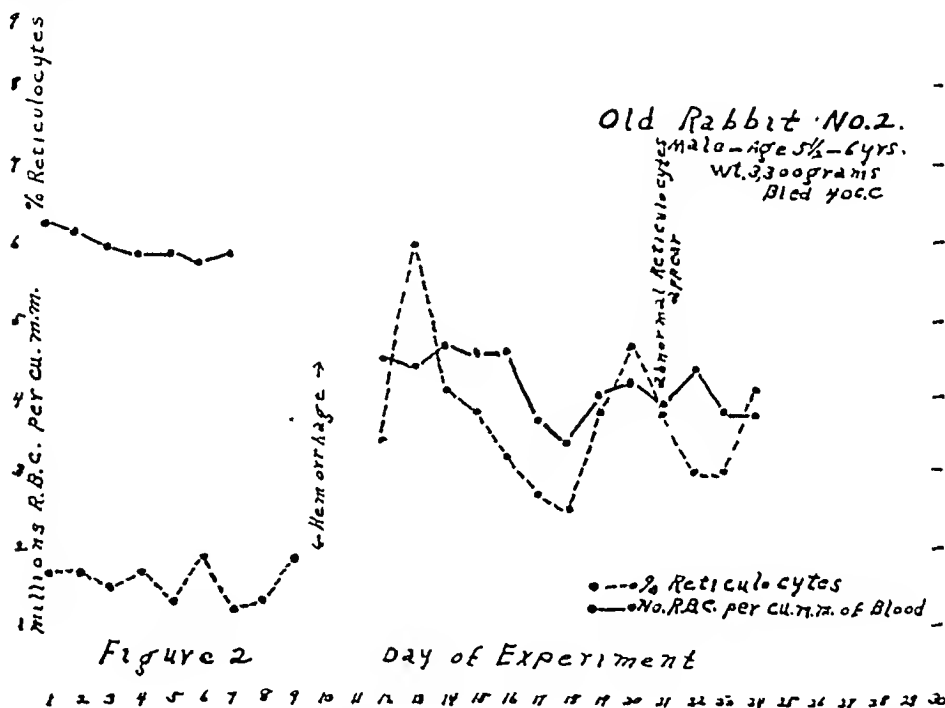


Fig. 2.—The per cent of reticulocytes and the total red corpuscle estimate were taken before and after a hemorrhage of 40 c.c. of blood, approximately one-fourth of its blood. These were taken on the day before the day of the hemorrhage and again after seventy-two hours. Two days after the hemorrhage two peaks of reticulocyte formation occurred on the 13th and 20th days of the experiment. The total red corpuscle count was lowest on the 18th day of the experiment. The average length of life of the red corpuscles can be estimated by counting the days between the 13th day and the 18th day. The average length of life of the red corpuscles was 5 days.

The hemoglobin was not tested in this experiment, since it was not deemed necessary. The control period lasted nine days. The average number of erythrocytes was six million, and the percentage of reticulocytes was 1.6 per cent. Tests were not made on the day of the hemorrhage, but they were made forty-eight hours later as well as on every subsequent day thereafter. On the twelfth day of the experiment, forty-eight hours after hemorrhage, there was a drop in erythrocytes to 4.5 millions and a rise in reticulocytes both in percentage and in actual number per cubic millimeter of blood.

The greatest percentage of reticulocytes occurred on the thirteenth day and the greatest destruction of erythrocytes occurred on the eighteenth day. The length of time between the peak of reticulocytes and the fall in the total number

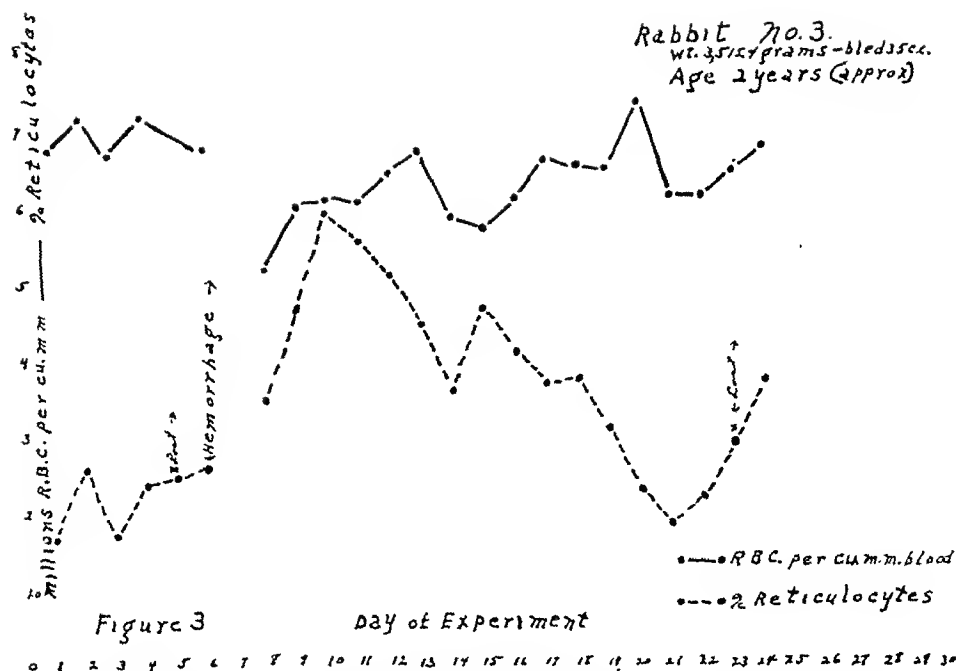


Fig. 3.—The per cent of reticulocytes and the total red corpuscle estimate were taken before and after a hemorrhage of approximately one-fourth of its blood. These were taken on the day of the hemorrhage and again after forty-eight hours. Two peaks of reticulocyte formation occurred on the 10th and 16th days of the experiment. The total red corpuscle count was lowest on the 15th and 22nd days of the experiment. The average length of life of the red corpuscles can be estimated by counting the days between the 10th and the 15th days and between the 16th and 22nd days, respectively. The average length of life of the red corpuscles was estimated as 5.5 days.

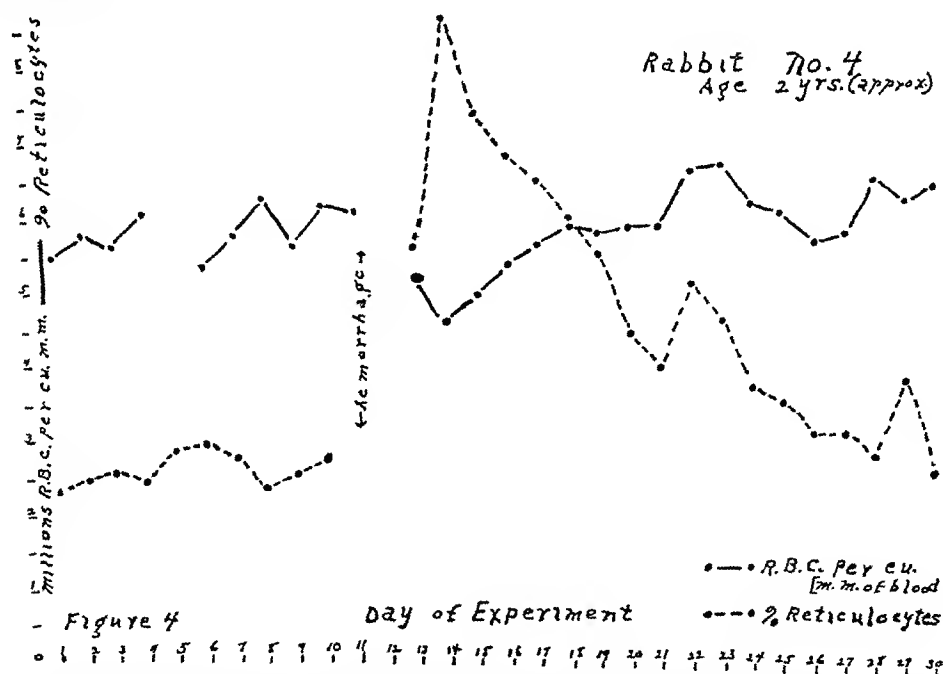


Fig. 4.—The per cent of reticulocytes and the total red corpuscle estimate were taken before and after a hemorrhage of approximately one-fourth of its blood. These were taken on the day of the hemorrhage and again after forty-eight hours. Two peaks of reticulocyte formation occurred on the 14th and 26th days of the experiment. The total red corpuscle count was lowest on the 19th or 20th and on the 26th or 27th days of the experiment. The average length of life of the red corpuscles can be estimated by counting the days between the 14th and 19th or 20th days and between the 22nd and 26th or 27th days. The average length of life of the red corpuscles was estimated as 5.5 days.

of corpuscles per cubic millimeter of blood was five days. This five-day period must be considered the average length of life of the red corpuscles of an old rabbit.

It will be noticed that the number of erythrocytes never returned to the normal level after hemorrhage.

*Middle-Aged Rabbits.*—The tables showing data for these two rabbits were omitted to save space, but the data of these tables were used in constructing the two graphs, Figs. 3 and 4, as well as Table III, where a comparison of all four rabbits was made. The average length of life of the corpuscles of each of these two rabbits was five to six days.

TABLE III

COMPARATIVE STUDY OF TOTAL BLOOD COUNT AND RETICULOCTE PERCENTAGE OF FOUR RABBITS OF DIFFERENT AGES

| RABBIT   | R.B.C. PER CU. MM. OF BLOOD (CONTROL PERIOD) | RETICULOCTE PERCENTAGE (CONTROL PERIOD) | AVERAGE LENGTH OF LIFE OF R.B.C. | R.B.C. MADE EACH DAY PER CU. MM. OF BLOOD | NO. OF RETICULOCTES ACTUALLY PRESENT AT ANY ONE TIME PER CU. MM. OF BLOOD (CONTROL) | DEGRADATION OF RETICULOCTE STAGE |
|--|--|---|----------------------------------|---|---|----------------------------------|
| Young rabbit<br>No. 1<br>Aged 6-7 mo.                                  | 6,400,000                                    | 2.4                                     | 6-7 days<br>Avg. 6.5 days        | 981,615                                   | 153,600   | 3 hr.<br>45 min.                 |
| Middle-aged rabbit<br>No. 3<br>Aged slightly less than 2 yr. (approx.) | 6,850,000                                    | 2.39                                    | 5-6 days<br>Avg. 5.5 days        | 1,245,451                                 | 163,715   | 3 hr.<br>10 min.                 |
| Middle-aged rabbit<br>No. 4<br>Aged 2 yr. (approx.)                    | 5,880,000                                    | 2.63                                    | 5-6 days<br>Avg. 5.5 days        | 1,069,000                                 | 137,000   | 3 hr.<br>4 min.                  |
| Old rabbit<br>No. 2<br>Aged 5½-6 yr.                                   | 6,000,000                                    | 1.6                                     | 5 days                           | 1,200,000                                 | 96,000  | 1 hr.<br>55 min.                 |

#### DISCUSSION—COMPARISON

The blood pictures of the young rabbit, Fig. 1, and the old rabbit, Fig. 2, were very similar during their respective control periods. The erythrocyte count of the young animal averaged 6.4 million, and the count of the old animal averaged 6 million. The reticulocyte percentage of the young rabbit averaged 2.4 and that of the old rabbit was 1.6. An amount of blood calculated to be approximately one-fourth to one-third of the total blood volume was removed from each animal at the end of the control period. Forty-eight hours were allowed to elapse before the daily tests were begun again.

The initial reticulocyte response of the young rabbit was greater and lasted over a longer period of time than that of the old rabbit, while the second peak of reticulocytes was higher in the old rabbit than in the young one. The time between the reticulocyte peaks for the young and old rabbits was nine and seven days, respectively. The explanation of this difference may be that the young rabbit produces enough reticulocytes during the first response to

bring the number of erythrocytes back to normal level, while the hemopoietic response in the case of the old animal was never sufficient to restore the original number of erythrocytes. Furthermore, more erythrocytes died at one time in the old animal and precipitated a higher second reticulocyte peak proportionately than in the case of the young animal.

On the ninth day after hemorrhage the young rabbit had its erythrocytes restored to a low normal, while, on the other hand, the old rabbit never returned to normal; in fact, it scarcely gained in number of erythrocytes at all.

Although the reticulocyte production was not complete enough and the cycle was not long enough before the subsequent intravascular hemorrhage occurred, due to a short length of life of the corpuscles, the conclusion reached was that the animal's red corpuscles decreased in number with each subsequent intravascular hemorrhage.

Seven days after the peak of reticulocytes in the young rabbit, there was a fall in total number of erythrocytes of approximately a million. In like manner five days after the peak of reticulocytes in the old rabbit, there was a fall in the total number of reticulocytes of a million or more. It cannot be doubted that the fall of a million in total number of erythrocytes in both animals on the eighteenth day of each experiment was due to an intravascular hemorrhage, a loss of erythrocytes born at the previous peak of reticulocyte formation. A second peak of reticulocytes, followed by an intravascular hemorrhage, was shown in the young rabbit. The period was between the nineteenth or twentieth days and the twenty-sixth day of the experiment.

Since the period of seven days in the young animal and the period of five days in the old animal must represent the average length of life of the red corpuscles in the two animals, the conclusion would be that the erythrocyte lives longer in the blood stream of a young animal than it does in the blood stream of an old one. This conclusion is further borne out in the results from two middle-aged (2 years old) rabbits in which the average length of life of the corpuscles was 5.5 days (see Table III).

The time between the hemorrhage and the first peak of reticulocyte response, five days and four days, respectively, in the young and old rabbits, was greater than the time between the intravascular hemorrhage and the succeeding reticulocyte response, two days each in young and old. The explanation, no doubt, is that the intravascular hemorrhage did not involve loss of volume; therefore, the time should be correspondingly decreased. Robertson and Boch (1919)<sup>14, 15</sup> have shown that it is the reduced hemoglobin per unit of blood due to dilution of the blood after hemorrhage that is the stimulating factor in reticulocyte formation.

Knowing the total number of erythrocytes per cubic millimeter of blood and the percentage of reticulocytes, the actual number of reticulocytes per cubic millimeter of blood can be determined:  $6,000,000 \times 1.6 \text{ per cent} = 96,000$  reticulocytes (see Table III, old rabbit).

Knowing the length of life of the red corpuscles, the number of corpuscles made each day may be determined:  $6,000,000 \div 5 = 1,200,000$  (see Table III, old rabbit).

Knowing the foregoing, the reticulocyte period can be determined:  $1,200,000 \div 96,000 = 1/12.5$  of a day, or one hour and fifty-five minutes (see Table III, old rabbit).

By similar calculation the reticulocyte stage in a young animal was found to last three hours and forty-five minutes. The comparative data are set out in Table III.

Calculations made for the two other rabbits shown in Table III are also tabulated. In practically every respect the blood pictures of the two, 2-year-old rabbits show conditions midway between the young and old rabbits.

The ability of the rabbit to recover from hemorrhage is due to a combination of the length of life of the corpuscle and of the number of corpuscles thrust into the blood stream in twenty-four hours. In the case of the young rabbit No. 1 the recovery was rapid because the length of life of the corpuscle is long and the number of reticulocyte percentage is high. The old rabbit No. 2 showed the other extreme, the reticulocyte percentage being low and the average length of life of the corpuscle short. It was unable to recover. Rabbits Nos. 3 and 4 recovered easily because the reticulocyte percentage was high, although the length of life of the corpuscles was somewhat shorter than in the case of rabbit No. 1.

Furthermore, the actual number of reticulocytes produced per cubic millimeter of blood every twenty-four hours was actually greater during the control period than that of the young rabbit; e.g., the young rabbit produced 984,615 reticulocytes per cubic millimeter of blood as compared to 1,200,000 produced by the old rabbit per cubic millimeter of blood.

#### CONCLUSIONS AND SUMMARY

1. After hemorrhage the reticulocyte cycles follow one another at regular intervals of a few days. The second reticulocyte cycle and each succeeding one is preceded by an intravascular hemorrhage in which corpuscles are destroyed but the volume of blood remains constant.

2. The longest period noted between reticulocyte cycles was nine days in a young rabbit, and the shortest period was seven days in an old rabbit.

3. The average length of life of the red corpuscle is equal to the period between the peak of a reticulocyte cycle and the end of the succeeding intravascular hemorrhage.

4. The average length of life of the red corpuscle of a rabbit varies inversely with age—the younger the rabbit, the longer the corpuscles live.

5. The average length of life of the red corpuscle varies from six to seven days in a young rabbit to five days in an old rabbit.

6. The extreme length of life of a red corpuscle may be much longer than the average life because many may be destroyed in "infancy" or in "early adult life."

7. The number of red corpuscles liberated into the blood stream each twenty-four hours by a young rabbit was 984,615 per cubic millimeter of blood, and by an old rabbit, 1,200,000 per cubic millimeter of blood.



8. The number of reticulocytes present per cubic millimeter of blood was 153,600 for a young rabbit, 96,000 for an old rabbit, and 1,245,451 and 1,069,000 for two middle-aged rabbits.

9. The duration of the reticulocyte stage was approximately four hours in a young rabbit and two hours in an old rabbit. The reticulocyte stage in the two other rabbits, aged 2 years, was about midway between that of the old and young animals; namely, 3 hours 10 minutes, and 3 hours 4 minutes, respectively.

10. An accurate picture of the activity of the red bone marrow can be obtained if the number of red corpuscles per cubic millimeter of blood, the average length of life of the red corpuscles, the per cent of reticulocytes present in the blood, and the duration of the reticulocyte stage of the red corpuscle are known.

I take this opportunity to thank Dr. William Moenkhaus and Dr. Paul Harmon, of the Physiology Department of Indiana Medical School, for help and encouragement.

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# THE EFFECT OF LOWERED TEMPERATURES UPON THE GROWTH OF THE FIBROBLAST IN VITRO: ITS APPLICATION TO WOUND HEALING\*

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IN THE course of tissue culture studies relating to the growth of embryonal and cancer cells under the influence of varying temperatures,† the behavior of the fibroblast has been so outstanding that it merits separate consideration. The vital importance of the role of this cell in wound healing and repair processes in general makes our understanding of its unusual resistance to cold and its adaptability to unfavorable environmental circumstances of especial significance.

At body temperature (37° C.) and under ideal culture conditions, in respect to material and media, the fibroblast multiplies rapidly and produces large, elongated, healthy appearing cells, with obvious coarse fibroglia fibers. It is not necessary to go into great detail regarding this aspect of the problem, as the behavior of the fibroblast under such circumstances has been thoroughly investigated by many competent observers.<sup>3, 4, 7, 10</sup> Like these other investigators we have found that the rate of such normal growth is not maintained for any considerable period in vitro unless the cultures are transplanted at frequent intervals. Otherwise the cells revert to what might be termed a "resting" or resistant phase, not unlike the encystment stage of certain parasites, only to grow again at a normal rate after a brief lag period when transferred to fresh media.

The resistance to lowered temperatures of the fibroblast is very marked. Initial congelation at 0° C. for as long as sixty hours does not significantly alter the growth capacity of these cells when the cultures are subsequently placed in the incubator at 37° C., other than for the usual lag noted previously in the tumor cell cultures. Nor is any inherent difference in the growth noted in parallel cultures with the temperature lowered gradually from an initial 37° C., as compared with those started off at the corresponding lower levels.

At 30° C. obvious differences in the appearance of the fibroblasts from those grown at 37° C. are seen. The cells are smaller both in respect to the size of the nucleus and the amount of cytoplasm. This decrease in size is especially prominent in respect to the length of the cell, the whole cell appearing more compact and sturdy, with ovoid rather than spindle nuclei.

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At 25° to 26° C. the fibroblasts are scarcely a third the size of those grown at 37° C. and show only little intercellular substance. Instead of migrating freely and extending in long fingerlike projections from the mother colony, they are arranged in almost solid radiating phalanx fashion, several cells deep, around the original explant. The cytoplasmic and nuclear detail is well defined in both unstained and vitally stained specimens.

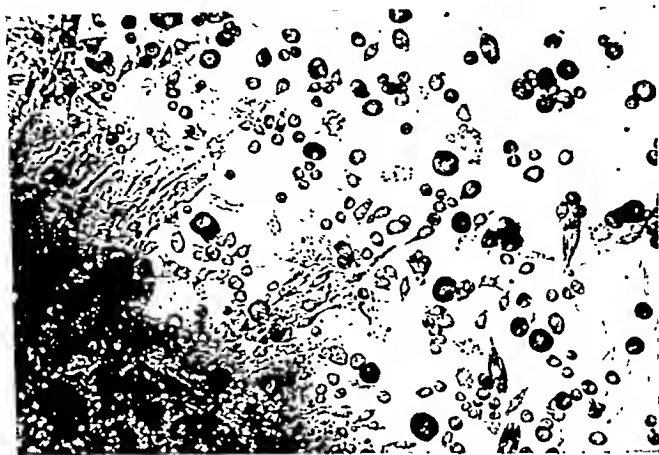


Fig. 1.—Normal mouse lymph node at 37° C. Approximate magnification,  $\times 150$ .

At 20° C. the rate of growth is still further retarded. Six or seven days elapse before the first evidence of new cell formation can be made out. At this temperature level the cells are likewise still further diminished in size, scarcely a fifth that of those grown at 37° C. They are very short and stubby in appearance, almost round or polyhedral rather than ovoid or spindled. As they slowly increase in numbers they occur in small sheets at the edge of the original inoculum, but it takes several weeks for this to occur.

At the 24° to 26° C. temperature levels the cells are far more resistant to environmental factors. This may be accounted for in several ways. Owing to their more compact arrangement they are less readily damaged physically by washing and changing their media. Owing to their slower rate of growth they survive in the original medium for a much longer period of time than the larger more rapidly growing fibroblasts at 37° C. This is undoubtedly due in part to their lower nutritional requirements, but also to the reduction in the metabolism of the cells, with a corresponding reduction in the concentration of the products of metabolism in the medium. This latter feature has been stressed by many writers, as well as ourselves in other communications, as being one of the very important factors which interfere with tumor cell culture. As part of this slowed metabolism the usual regressive degenerative changes of the cytoplasm of cells grown "in vitro" are obviously retarded, which in turn fits in with the previous comments in helping to explain the increased resistance of these cells to lowered temperatures.

We believe that these observations are well established and might be expressed almost mathematically because of their constancy in the case of literally

hundreds of such tissue explants. There is a direct proportional relationship not only between the temperature and the rate of cell growth, but also in respect to temperature and the size of the cell, its degree of differentiation, its metabolic activity, and its resistance to environmental circumstance. On the basis of these studies a temperature range of  $25^{\circ}$  to  $30^{\circ}$  C. would seem to be optimal in respect to wound healing. Under such circumstances one logically might expect that the healing process would be somewhat slower than at body temperature, but that the amount of collagen production, with its subsequent ugly contraction and scar formation, would be reduced appreciably.

That this optimal reaction actually does occur clinically was shown by Smith and Fay<sup>12</sup> incidentally in their early studies concerning the effect of hypothermy on cancer cell growth. In one case, especially, of extensive ulcerative breast carcinoma treated by continuous local hypothermy for nearly six months, not only did the tumor disappear, but the ulcerated surface healed over completely with soft scar tissue having the consistency of normal skin. Histologic study of biopsy material from this and numerous other cases confirmed the clinical impression. The tissues showed a connective tissue relatively rich in nuclei and with a minimum of dense collagen formation.



Fig. 2.—Normal mouse lymph node at  $30^{\circ}$  C. Approximate magnification,  $\times 150$ .

The recent work of Allen<sup>1</sup> and his associates in the healing of diabetic and traumatic amputation stumps under the influence of cold is another illustration of the clinical application of this principle. In almost every instance healing by first intention resulted, and with a minimum of scar production. The repair process, as might be expected, was quite regularly delayed, requiring three to four weeks instead of the usual ten days to two weeks. On the other hand, the wounds did not break down, and the stumps presented a much more satisfactory end result for subsequent artificial limb appliances. As part of the mechanism involved in the use of hypothermy for such wound repair, it should be noted that the external temperatures employed ( $5^{\circ}$  to  $10^{\circ}$  C.) are bacteriostatic, and thus the danger of secondary infection is almost eliminated. It should be further mentioned that actual freezing must be avoided, and that the penetra-

tion of the cold into the deeper tissues from local external application drops off with almost algebraic progression, so that the desired levels of  $20^{\circ}$  to  $25^{\circ}$  C. are actually present in the deeper tissues of the wound where the connective tissue repair process is going on.

On the basis of such clinical observations and the confirmatory laboratory evidence of the behavior of the fibroblast at these lower temperatures, the more widespread use of hypothermy in wound healing would seem to be warranted. Its application in plastic surgery, of the cosmetic type particularly, comes to mind immediately. The possible prevention of keloid formation is another field which might profitably be explored. Its place in clearing up old osteomyelitis lesions might likewise be investigated.

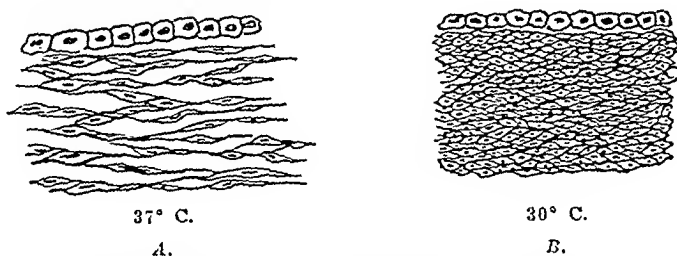


Fig. 3.—Schematic representation of fibroblastic proliferation at A,  $37^{\circ}$  C. and B,  $30^{\circ}$  C. Note the smaller size, compactness, and diminished intercellular products at the lower temperature.

It is of interest that these various tissue culture studies and clinical observations of ours are indirectly confirmatory of the fundamental work of Burrows<sup>4</sup> on wound healing, although the approach to the problem is from a wholly different angle. Burrows noted that the growth of the fibroblast in his experimental wounds at body temperature was most profuse in the central areas which were less well vascularized than in the periphery of the lesion. He advanced the hypothesis that the fibroblasts grow better when they are closely packed together so that certain metabolic products of oxidation accumulate in optimal amounts, through their inability to be absorbed and carried away by an inadequate blood supply. By corollary he comments that if the wound is washed too frequently, the rate of growth of the fibroblasts is likewise reduced, and healing is thereby delayed and less satisfactory.

In our own studies the reduction of temperature to  $25^{\circ}$  or  $30^{\circ}$  C. gives us similar optimal conditions for satisfactory wound healing. The cells are growing vigorously; they are closely packed and the circulation is slowed. Thus the products of metabolism are in close contact with the cells, and remain so, through the inability of the slowed blood stream to carry them away. In addition these temperatures are bacteriostatic to a very considerable degree and aid materially in checking any infection which might be present (Fig. 3).

Finally, there is a definite relationship between epithelialization and fibroblastic proliferation in a wound. When the surface becomes covered with epithelium, the stimulus to fibroblastic proliferation is lost. If this epithelialization is accomplished too rapidly, the underlying granulation tissue tends to undergo regressive changes, the collagen contracts, and we have a depressed secondary

contracted scar. If, on the other hand, the repair process is slower, the connective tissue is adequate in amount, compactly arranged, with relatively little collagen, and the final scar is minimal with little or no retraction. From our observations all of these prerequisites for good wound healing seem to be realized by temperatures between 25° and 30° C.

#### SUMMARY

The behavior of the fibroblast in tissue culture at varying temperatures ranging from 0° to 37° C. is recorded. An optimal level of around 25° C. is suggested as of practical value in wound healing.

Clinical observations of the use of hypothermy in various fields, including a review of some of the more recent literature, are presented.

A plea for the wider use of local hypothermy in various clinical problems relating to wound healing is made.

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## AN UNUSUAL P-WAVE IN CHEST LEAD CF<sub>2</sub> FOLLOWING SPONTANEOUS PNEUMOTHORAX\*

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ALTHOUGH it is well recognized now that changes in the position of the heart will alter the electrocardiogram, little work has been done to illustrate these changes in the chest leads. Recently we have had the occasion to follow the changes in the electrocardiogram caused by a spontaneous pneumothorax which occurred in a patient who had an abnormal electrocardiogram due to congenital heart disease. The electrocardiographic changes were correlated with changes in position of the heart as the pneumothorax appeared and then resolved. A control record was available before the pneumothorax developed, and serial electrocardiograms were taken during the course of the patient's illness.

A striking alteration in the P-wave of chest Lead CF<sub>2</sub> was especially noted. The changes in this P-wave were similar to, but more marked, than those reported by Gertz<sup>1</sup> from this department. This record serves to emphasize again that such P-wave changes are suggestive of a position change of the heart.

### CASE REPORT

The patient was a 19-year-old white male, who entered Michael Reese Hospital on the service of Dr. W. A. Brams on Nov. 12, 1939, complaining of cough and pain in the chest of one day's duration. He was known to have had heart disease since birth, and his activities had always been restricted. He had been followed in the out-patient clinic.

On the morning of admission he was awakened by pain in his right chest and a nonproductive cough. On two occasions following a paroxysm of coughing, he became dyspneic and perspired profusely. There was no precordial pain or hemoptysis.

His past medical history revealed the presence of cyanosis since birth, pneumonia at the age of 5, measles, and chickenpox. One brother had rheumatic heart disease. Three other siblings, his mother and father, were all alive and well. He had had a series of electrocardiograms taken before this episode, one of which is shown in Fig. 1A.

On admission he had a temperature of 99° F., a pulse rate of 120, and a respiratory rate of 28 per minute. The blood pressure was systolic 96 and

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diastolic 70. The patient was underdeveloped and thin. His skin was cyanotic. He was slightly dyspneic even with the back rest elevated at a 60 degree angle. There were no petechiae visible. The right hemithorax appeared larger than the left, and it moved less with respiration. Tactile fremitus was diminished, and the percussion note was hyperresonant over the entire right side of the chest. Breath sounds were absent, except for a small area of bronchial breathing beneath the clavicle. The pulmonary findings on the left side were normal. The apex beat was diffusely palpable in the fourth, fifth, and sixth left intercostal spaces as far laterally as the anterior axillary line. There was no cardiac dullness to the right of the sternum. The heart rhythm was regular, and the tones were loud. There was an accentuation of the first sound at the apex. No murmurs were audible at the time of this examination. The abdomen was negative. There was a moderate left thoracic kyphoscoliosis. Clubbing of the fingers and toes was marked. The diagnosis was congenital heart disease and right spontaneous pneumothorax.

An electrocardiogram was taken at this time (Fig. 1B). A teleoroentgenogram also taken on the day of admission showed a right pneumothorax with almost complete collapse of the upper and middle lobes against the mediastinum. The right lower lobe appeared 80 to 90 per cent collapsed. There was a slight upper dorsal scoliosis so that it was difficult to determine whether there was a shift of the heart or mediastinum to the left.

On Nov. 13, 1939, the venous pressure was 6.8 cm. of water, the circulation time (arm to tongue) by the calcium gluconate method was 15.5 seconds, and the vital capacity was 1,300 c.c. The intrapleural pressure on the right side varied between 0 and +4.0 cm. of water on quiet respiration. After 1,000 c.c. of air were aspirated from the right chest, the foregoing determinations were repeated. The vital capacity rose to 1,600 c.c., and the intrapleural pressure varied from -6.0 cm. to 0 cm. of water during respiration. Electrocardiograms were taken before and after aspiration of the air and are shown in Fig. 2, A and B. After aspiration Lead CF<sub>2</sub> was recorded with the patient in three different positions: (1) sitting up, (2) left lateral, and (3) right lateral (Fig. 3).

A chest film twelve hours after the aspiration of the air showed little change in the findings. Blood studies revealed an intense secondary polycythemia with a red blood cell count of 6,720,000, hemoglobin, 24.0 Gm. per 100 c.c. of blood, volume index of 1.1, color index 1.05 and a hematocrit of 71 per cent. Fluoroscopic examination on November 18 showed a persistence of the pneumothorax, with an almost total collapse of all three lobes of the right lung. The heart was slightly deviated to the left, with a rounded contour of its left border. The right ventricle was moderately enlarged in the right oblique. There was no left auricular or ventricular enlargement. The aortic knob was hardly visible.

The patient's course in the hospital was uneventful. Fluoroscopy on November 25, thirteen days after admission, showed 60 to 70 per cent collapse of the right lung still present. Another electrocardiogram was taken at this time (Fig. 1C). On December 4, twenty-two days after admission, 50 per cent collapse was observed on fluoroscopic examination. One week later almost all



physical signs of pneumothorax had disappeared. A loud systolic murmur at the apex transmitted toward the base was now audible. The remainder of the physical findings were unchanged. The possibility of a patent interventricular septum, moderate overriding of the aorta to the right ventricle, and narrowing of the infundibulum of the pulmonary artery were considered as the anatomic defects.

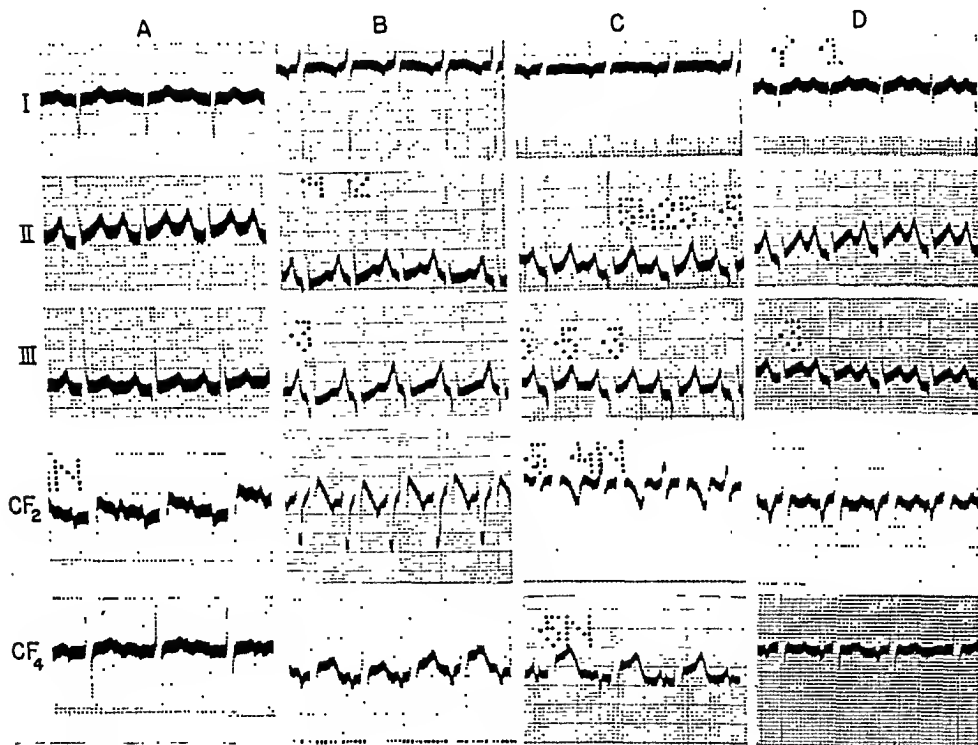


Fig. 1.—Effect of pneumothorax and its spontaneous resolution on the electrocardiogram. Segment A, control record taken on Jan. 9, 1939. Segment B, record taken on Nov. 13, 1939, after a spontaneous right pneumothorax. (Note the deeply inverted P-wave in Lead CF<sub>2</sub>.) Segment C, record taken on Nov. 25, 1939, when the right lung was 60 to 70 per cent collapsed. Segment D, record taken on April 6, 1940, when all evidence of the pneumothorax had disappeared. See text for discussion.

The patient was discharged on December 12, thirty days after admission, much improved. Further observations were made in the out-patient department. A teleoroentgenogram was taken on Feb. 3, 1940, and showed no evidence of a pneumothorax. A final electrocardiogram was taken on April 6, 1940 (Fig. 1D). His course since discharge has been essentially similar to that before the pneumothorax.

#### DISCUSSION

Examination of Fig. 1 shows the effect of the pneumothorax upon the electrocardiogram. Record A, the control, is abnormal, showing a right ventricular preponderance and peaked P-waves in the limb leads. Following the pneumothorax (segment B), marked changes in the S-T segment and T-wave were produced, and there was an increase in the axis deviation to the right. The most striking changes occurred, however, in the P-waves, and especially the

P-wave in Lead  $CF_2$ ; this deflection consisted of a deeply inverted wave 14 mm. in amplitude, preceded by a tiny preliminary upright phase. With partial resolution of the pneumothorax, segment 1', the electrocardiogram tended to revert toward its previous appearance and P-wave changes occurred in all leads. The P-wave in  $CF_2$  was still large, but it had become diphasic with the first phase up. The record, segment D, taken after all evidence of the pneumothorax had disappeared is fairly similar to the control record taken before the

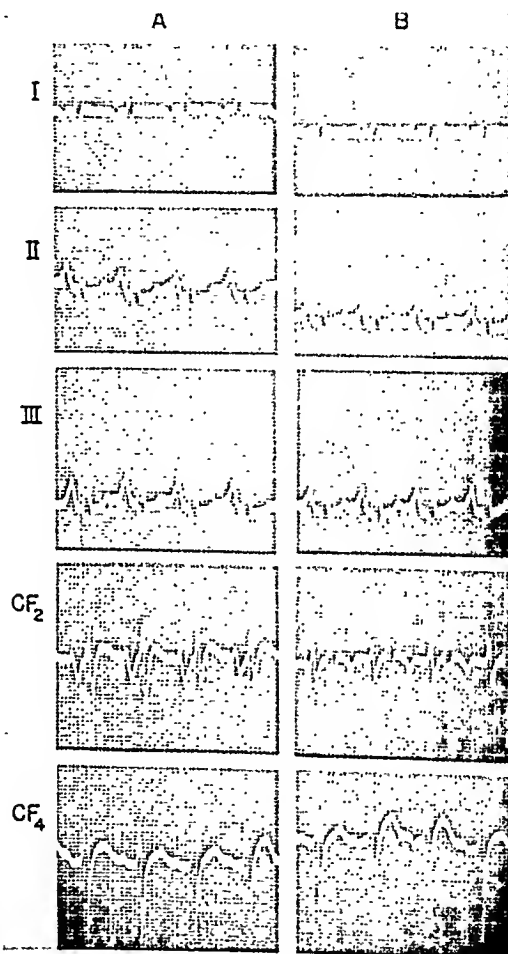


Fig. 2.—Effect of partial release of pneumothorax on the electrocardiogram. Segment A, control record (compare with segment B, Fig. 1). Segment B, record taken immediately after 1,000 c.c. of air had been withdrawn from the right pleural cavity. Note the decrease in the right axis deviation and the marked change in the P-wave in Lead  $CF_2$ .

pneumothorax developed. It is, therefore, proper to ascribe the electrocardiographic alterations in this case to the pneumothorax. This statement is further supported by comparing the similarity in the changes which occurred immediately following the withdrawal of 1,000 c.c. of air from the chest (Fig. 2) and the more gradual changes illustrated in Fig. 1 (B, C, and D) as the process resolved spontaneously.

The P-wave in Lead  $CF_2$  in these figures, although larger in amplitude, is similar to the one described by Gertz.<sup>1</sup> It is the type of deflection obtained by

Wilson, Macleod, and Barker<sup>2</sup> and others when a unipolar lead with one electrode connected directly to the auricle is applied. However, the phase relations of the P-wave in this case and in that of the authors just mentioned are mirror images because the lead connections to the galvanometer were reversed.

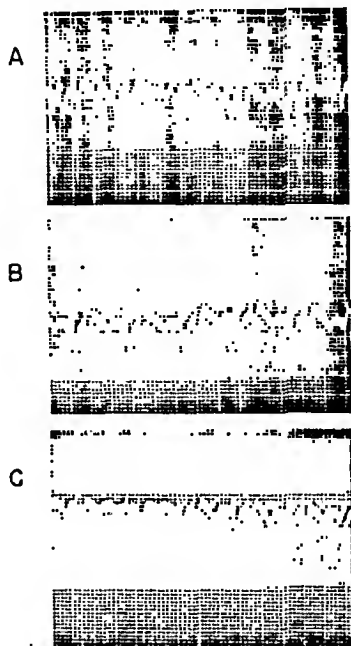


Fig. 3.—Records showing effect of postural changes in the electrocardiographic contour of the chest Lead  $CF_2$  in this patient when the pneumothorax was present. Segment A, patient sitting upright. Segment B, patient in the left lateral recumbent position. Segment C, patient in the right lateral recumbent position. (Note the marked P-wave changes and the variations of the P-wave with respiration in segment C.)

The P-wave changes in this case must be ascribed to a displacement of the heart, such that the auricle comes to lie beneath the chest electrode. When the pneumothorax was at a maximum, viz., in Fig. 1B and Fig. 2A, the appearance of the P-wave suggests that the part of the auricle in which the pacemaker of the heart is located was lying close to the chest electrode. Such records, or rather their inverted images, because of the reversed electrode connections, have been obtained with unipolar leads in the neighborhood of the sinus node. In order for these P-wave changes to occur, it must be presupposed that the heart is pushed over laterally, ventrally, and caudally, and that it is also rotated in a counterclockwise direction when viewed from above. Counterclockwise rotation causes a deviation of the electrical axis to the right (Katz and Robinow<sup>3</sup>); this was observed in our case.

With resorption of the pneumothorax, the displacement of the heart was less marked. Hence the region of the sinus node became farther removed from the chest electrode, and at this stage a P-wave with more nearly equal phases was recorded (Figs. 1C and 2B).

As might be expected, the heart in a chest with a partial pneumothorax is more mobile than normal. This is shown by the changes in the contour of the electrocardiogram in Fig. 3 when the body posture was altered. In addition,

the respiratory variation in the P-wave contour was greatest when the patient was in the right lateral position with his heart supported from below by the collapsed lung and the pneumothorax (Fig. 3C). These shifts in the heart's position alter the relation of the heart to the good and poor electrical conductors in contact with its surface and lead to the variations in the electrical field. It has been shown that the conductors surrounding the heart vary in their ability to conduct the electric currents generated by the heart and that alterations in these contacts affect the electrocardiogram (Katz and Korey;<sup>4</sup> Lindner and Katz<sup>5</sup>).

The view presented by Gertz<sup>1</sup> is thus confirmed and amplified: namely, that peculiar large P-waves in the chest Lead CF<sub>2</sub> may be a sign of cardiac displacement in which the right auricle is brought into close proximity with the chest electrode.

#### SUMMARY

A case is presented showing an unusual and deeply inverted P-wave of 14 mm. in Lead CF<sub>2</sub>. This P-wave was produced by cardiac displacement, resulting from a spontaneous pneumothorax which brought the right auricle into close apposition to the anterior chest wall. The contour of the P-wave was such as to suggest that the part of the auricle beneath the chest contained the pacemaker, the sinus node. As the pneumothorax was relieved, the large P-wave became diphasic, suggesting the apposition of other parts of the auricle to the anterior chest wall. Finally, as further resolution of the pneumothorax progressed, the P-wave returned to its smaller and more nearly normal appearance, which existed before the pneumothorax occurred. During the period of pneumothorax, the changes in the P-wave with change in body posture indicated greater than normal mobility of the heart.

We are grateful to Dr. L. N. Katz, at whose suggestion this study was undertaken, for his advice and guidance.

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# CLINICAL CHEMISTRY

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## PLASMA ALBUMIN, GLOBULIN, AND FIBRINOGEN IN HEALTHY INDIVIDUALS FROM BIRTH TO ADULTHOOD\*

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### II. "NORMAL" VALUES

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THE literature records several studies of the plasma proteins in "normal" individuals. These values are not wholly adequate, since most of the studies have been made upon small groups of persons, chiefly adults, whose state of health in many cases is open to question. There is a great paucity of data with respect to plasma protein values in infancy and early childhood. In addition to this lack of adequate material, the existing studies have been based upon different methods of analysis which do not give comparable results. The many and varied functions attributed to the different protein fractions of the blood plasma have made it increasingly important that a quantitative study be undertaken to determine the amount of albumin, globulin, and fibrinogen present in the blood of healthy individuals. Such a study also furnishes the necessary foundation for the interpretation of findings in pathologic conditions. We have determined the concentration of the protein fractions in the plasma of 547 healthy persons by means of a salting-out separation of the different proteins, followed by Kjeldahl analysis. The details of our analytical procedure have been given in a previous publication.<sup>1</sup>

#### MATERIAL USED

The subjects for this study were obtained from various sources. A few of the children were personal friends of ours, or children of the Child Research Council. The majority of the babies and younger children were from maternity hospitals or Homes at which they received regular medical supervision and in which the dietary was considered to be adequate. The adolescents were students at West Denver High School and were examined and questioned by a physician and one of us when the blood was taken. The adults were medical students and laboratory workers. In every case an attempt was made to rule out those who might present a clinical or subclinical variation in any of the protein fractions as a result of recent immunization, malnutrition, dehydration,

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chronic or acute disease, or recent injury. Samples were not taken from persons presenting a history of mild illness within the preceding two weeks, or of immunization, severe infection, or contagion within the preceding three months. With two exceptions, the subjects under one year of age were all considered to have been full-term babies. Two babies, one 3 months old and the other 6 months old when the blood was taken, were probably premature at birth. A few persons who had minor injuries, such as blisters or scratches of the hands or feet, were included. A Wassermann test was done on the serum of each child from the institutions, and those few with a positive test have been omitted from the present study. The majority of the children had also been tested with tuberculin at regular intervals while in the Homes. A few (eight) positive tests were obtained, but inasmuch as these showed no clinical or x-ray evidence for active tuberculosis, they have been included.

It is conceivable that each disease to which a child becomes immunized might leave a permanent record of some increase in the total plasma globulin, since the antibodies are found in this fraction. Complete histories were not available on the persons of this study, and therefore, no attempt has been made to classify them on this basis.

Most of the blood samples were taken during the morning. With older children and adults, the majority of the samples were obtained from one-half to two and one-half hours after the usual breakfast. Twenty-six analyses on children between 3 and 9 years of age were made on bloods taken after a fast of twelve hours. No consistent difference was found in any of the protein fractions between the children who had fasted and other children of the same age. Similar findings have been reported by other workers.<sup>2,7</sup> With the small infants, blood samples were also obtained at varying periods of time after feeding. No separation has been made between those who were entirely breast fed and those who received supplemental feedings. In the period from the third day of life to the end of the first month, 90 babies were known to be entirely breast fed, and 15 received one or more supplemental bottle feedings each day. After the first month most of the babies were wholly or partly bottle fed. The small number of bottle-fed babies in the first month of life and of breast-fed babies in the older age groups makes it impossible for us to say with certainty whether the type of feeding is a factor in determining the plasma protein level in infancy; however, no definite association between the type of feeding and the levels of the different protein fractions has been observed in our series. This is in contrast to the work reported by Ujsaghy,<sup>8</sup> in which lower values for the total plasma protein were found in breast-fed infants as compared with artificially-fed infants of the same age.

Blood samples were taken from the external jugular, the femoral, or the scalp veins in the small infants, and from the arm or hand veins in the larger children and adults. In taking the blood, a minimum amount of stasis was used. The blood sample (2 to 4 c.c.) was placed immediately in a tube containing a slight excess of dry heparin (about 0.5 mg.), mixed, and centrifuged in a stoppered tube as quickly as possible. The plasma was separated usually within one hour from the time the blood was taken, and in all cases, within three hours.

## RESULTS

Variation in the plasma concentration of the different protein fractions has been determined within groups of similar subjects. In addition, we have attempted to determine the association, if any, between the different protein concentrations and the factors of age, sex, and season of the year. The series of 566 determinations on 547 persons is made up of 303 determinations on males and 263 on females. These are distributed equally throughout the entire age sequence, except in the range above 20 years, in which the females are predominant; and at 11 years and below 6 months, where the number of males is in excess. The blood samples have been separated into two groups according to the season in which they were done: "summer" extending from April 1 through October 31, and "winter," from November 1 through March 31. In the age period from one month to 6 months, the grouping according to season is uneven. From 6 months to 12 years the analyses are more evenly divided between winter and summer for each age interval and for each sex. For the group from 12 to 20 years, most of the analyses were done during the winter, and from 20 to 40 years, in the summer. The complete distribution of the analyses is summarized in Table I.

TABLE I

DISTRIBUTION OF ANALYSES ACCORDING TO SEASON OF YEAR, SEX, AND AGE OF PERSONS

| AGE                  | NUMBER OF ANALYSES |        |         |        |
|----------------------|--------------------|--------|---------|--------|
|                      | MALES              |        | FEMALES |        |
|                      | WINTER             | SUMMER | WINTER  | SUMMER |
| Birth to 15 days     | 27                 | 18     | 16      | 13     |
| 15 days to 31 days   | 19                 | 15     | 8       | 11     |
| 1 month to 6 months  | 34                 | 9      | 20      | 6      |
| 6 months to 4 years  | 20                 | 26     | 22      | 18     |
| 4 years to 8 years   | 16                 | 25     | 14      | 29     |
| 8 years to 12 years  | 22                 | 26     | 12      | 25     |
| 12 years to 20 years | 25                 | 15     | 34      | 8      |
| 20 years to 40 years | 2                  | 4      | 6       | 21     |

Limitations of space prevent a detailed presentation of data; however, these have been analyzed by accepted statistical procedures (Mainland<sup>9</sup>) and the significant findings are given.

*Albumin.*—The albumin fraction shows no significant variation between the sexes, nor with the height, weight, or body surface of the subjects, but it does vary with age and with the season of the year. During the first month of life the albumin is low, the mean value being 3.79 Gm. per 100 e.e., with a standard deviation of  $\pm 0.33$  Gm. There is no apparent change with age at this early period, but the variation between different babies of the same age is large. At about one month a rise in the albumin with increasing age becomes apparent. This continues until the "adult" level is reached, sometime between 6 months and 1 year, after which there is no further significant change with age. As pointed out, the babies in our series were predominantly breast fed in the first month of life and bottle fed after this age. It, therefore, appears possible that the increasing plasma albumin concentration may be associated with the change in diet and may not be entirely referable to age. A consideration of the 90

breast-fed and 15 bottle-fed infants under one month of age gives no indication of a relationship between the plasma albumin level and the type of feeding; however, a larger group of infants must be studied before such a relationship can be determined definitely. In our series there is an apparent rise in the plasma albumin level between the ages of 13 and 20 years old. This may result from the fact that this group of analyses was done during the winter, and the rise in values may be ascribed to seasonal variation rather than to a change with age. The mean "adult" level is 4.70 Gm. per 100 c.c., with a standard deviation of  $\pm 0.32$  Gm.

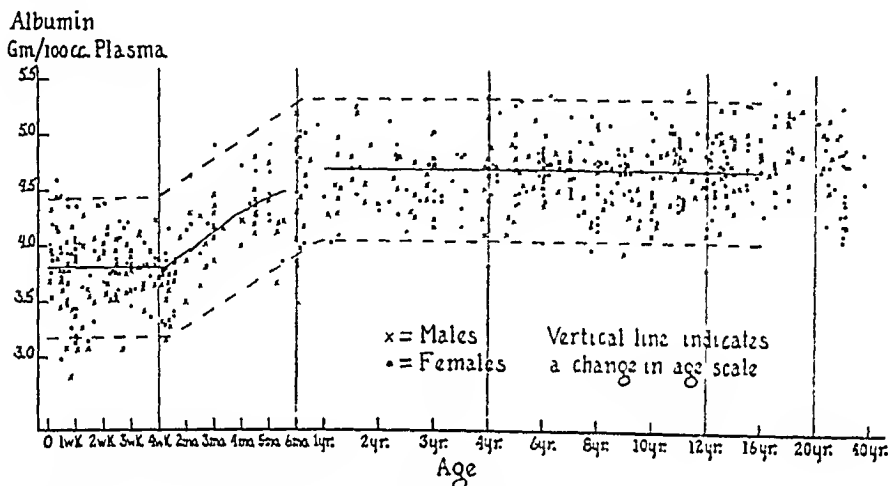


Chart 1.—A scatter diagram of albumin, expressed as grams per 100 c.c. of plasma, referred to age. The unbroken line indicates mean values; the region between the broken lines is that included within plus and minus two standard deviations from the mean.

We have determined the trend of values during the period of change. This is shown graphically in Chart 1, together with the values for both younger and older persons. It can be seen that the spread covered by two standard deviations, including about 95 per cent of the cases, is very large for all ages. This spread is made up of two components, the true biologic variation and the variation caused by chance errors in the analytic procedure. The true biologic variation has been calculated as follows:

$$\begin{aligned}
 \text{S.D.}_B &= \sqrt{(\text{S.D.}_T)^2 - (\text{S.D.}_M)^2} \\
 &= \sqrt{0.32395^2 - 0.07705^2} \\
 &= \sqrt{0.099007} \\
 &= \pm 0.314
 \end{aligned}$$

The symbol "S.D." represents the standard deviation, and the subscript letters "B," "T," and "M" refer to the deviation characterized as biologic, total, and measurement, respectively. It is seen that the true variation between individuals is large, even after allowing for the increment caused by errors of measurement. By means of longitudinal studies we hope to determine whether this wide spread indicates that the plasma albumin concentration varies widely in a given person from time to time, or maintains itself within the individual, at a high or low level in relation to other persons.



The albumin analyses have been studied with respect to seasonal variation. In the series of 127 babies up to one month of age, there is no significant difference in the plasma albumin level at different seasons of the year. For persons over 3 years of age, 142 winter samples give a mean value of 4.83 (S.D.<sub>r</sub> =  $\pm 0.30$ ), and 167 summer samples show a mean of 4.59 (S.D.<sub>r</sub> =  $\pm 0.31$ ). Although this difference is not great, the probability that it would occur by chance alone is much less than 1 in 100. Chart 2 shows the frequency distribution of albumin values in the two groups. With the data available we have not been able to determine at what point between one month and 3 years of age the seasonal variation appears.

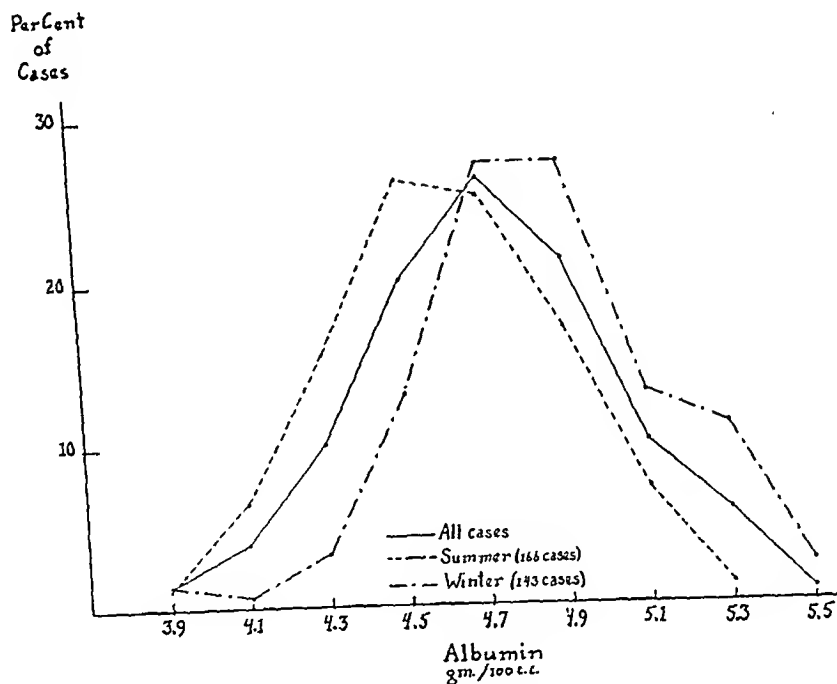


Chart 2.—Winter and summer frequency polygons of albumin values in 309 analyses on individuals over 3 years of age.

Bazett, Sunderman, Doupe, and Scott<sup>10</sup> have presented evidence to show that during the transition from winter to summer there is an increase in blood volume in human subjects and a corresponding decrease in total plasma protein concentration. As the warmer temperature is maintained, the proteins increase until the initial level is reached. During the transition from summer to winter, the reverse set of changes takes place. It might at first seem that this type of mechanism could account for the seasonal variation in albumin which we have observed, since our "summer" group would, to a large extent, include the transition from winter to summer, and the "winter" group would demonstrate the opposite change. However, since the globulin and fibrinogen have not shown a seasonal variation similar to that observed in the albumin fraction, the picture is more complex than one of simple dilution and concentration of the plasma.

Yosida<sup>11</sup> has studied rabbits with respect to the seasonal variation in certain plasma constituents. Although it is difficult to interpret his findings in terms of absolute values for the protein constituents, the figures given should be comparable within themselves. His results on this species of animal show a higher albumin during the summer months than during the winter. This is in direct contradiction to our results in which the lower albumin values occurred during the summer. It seems possible that different species of animals might respond differently to temperature changes. It is also possible that the variation which we have observed may be the result of changes in diet and living habits rather than temperature changes, whereas in the case of rabbits, the diet, activity, and living conditions are more subject to control.

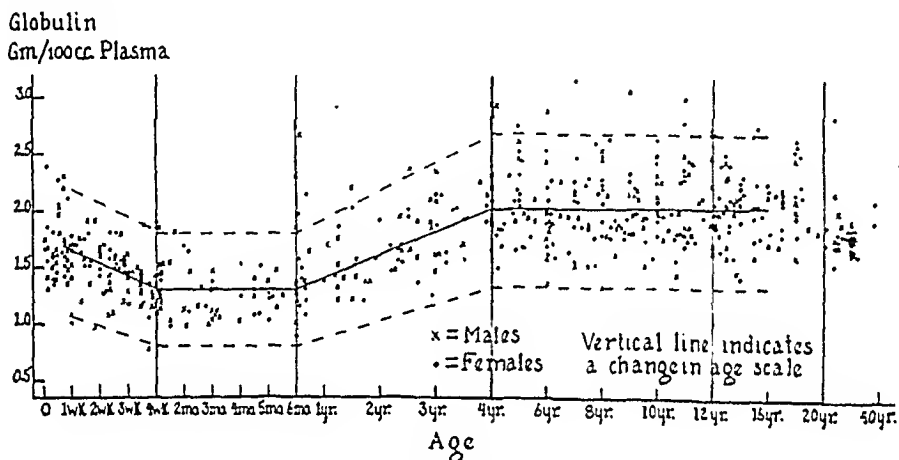


Chart 3.—A scatter diagram of globulin, expressed as grams per 100 c.c. of plasma, referred to age (see legend of Chart 1).

**Globulin.**—The plasma globulin concentration has been found to vary with age, as shown in Chart 3, but it does not vary with sex or size of the person, nor with the season of the year. At birth, and for the first week of life, the globulin is present in a mean concentration of 1.66 Gm. per 100 c.c., with a standard deviation of  $\pm 0.29$ . Between the first and the fifth week the value falls to 1.31 Gm. per 100 c.c. ( $S.D._T = \pm 0.25$ ), where it is maintained until the age of about 6 months. At about 6 months a gradual rise in globulin concentration becomes apparent, and continues to an age of about 4 years, at which time the "adult" level of 2.03 Gm. per 100 c.c. ( $S.D._T = \pm 0.34$ ) is reached. After 4 years, up to 20 to 40 years, there is no significant change in the globulin level. In Chart 3 there appears to be a fall in the globulin in the period between 20 and 40 years. This is probably not a real change, but it may result from the fact that 15 determinations in this age group were obtained on two individuals who have maintained a consistently low globulin over a period of many months.

As was the case with the albumin, the greater part of the variation in values at any age level is apparently a true biologic variation, and not the result of chance errors of analysis. For all persons over 5 years of age the mean globulin concentration is 2.03 Gm. per 100 c.c.; the standard deviation representing biologic variation ( $S.D._B$ ) is  $\pm 0.34$ .

The distribution of values shows a definite skew toward the higher globulin concentrations, as shown in Chart 4. Similarly, there are more above the area covered by plus or minus two standard deviations than there are below it, as can be seen in Chart 3. There is no known explanation for this fact; however, it seems possible that in spite of the care exercised in the selection of subjects, a few may have been included who show an elevated globulin as a result of infection or immunization. It is known<sup>12-15</sup> that the development of antibodies is frequently accompanied by an increase in some fraction of the total globulin, but little is known of the duration of this hyperglobulinemia.

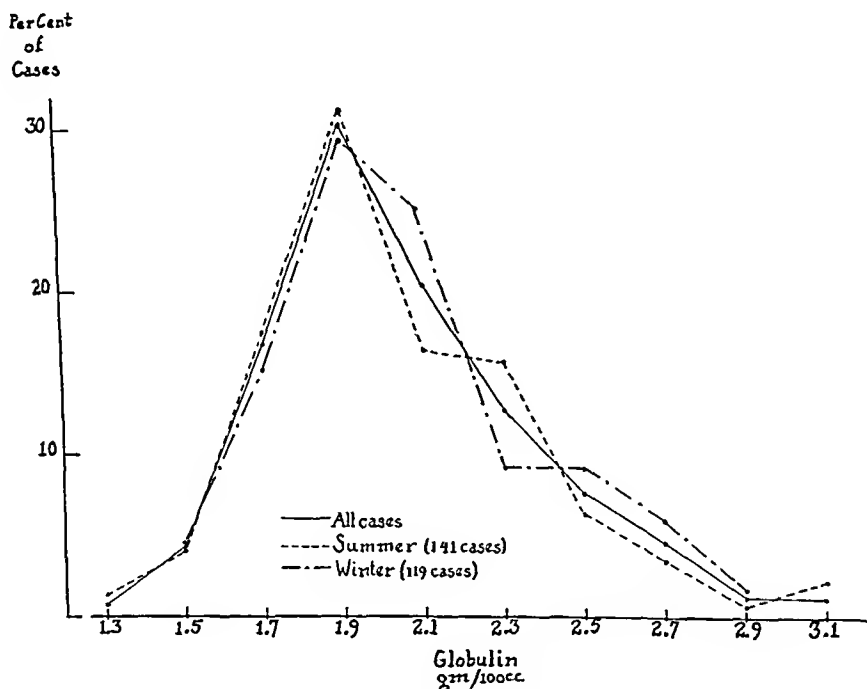


Chart 4.—Frequency polygon of globulin values in 260 analyses on persons over 5 years of age.

*Fibrinogen.*—The most striking feature of the findings with respect to fibrinogen is a wide scatter of the values in all groups. Chart 5 shows the distribution of values for both sexes graphed with respect to age. From the chart it will be observed that there is a slight tendency toward a higher mean value and greater variation at birth than in the older age groups. This may or may not be a real change; in any event it is probably too small to be of clinical significance, except in so far as it may indicate instability of the plasma fibrinogen concentration in the first weeks of life. After one month there is no significant change with age in the mean level of plasma fibrinogen. This value is 0.21 Gm. per 100 c.c., with a standard deviation (total) of  $\pm 0.0590$ . As was the case with the albumin and globulin fractions, the spread in values seems to be due to a true biologic variation, the standard deviation corrected for chance errors of analysis being 0.0585.

In the period up to 5 years of age there is no significant difference between the sexes in the fibrinogen level. After this age the males tend toward lower values, the mean for 123 males being 0.201 (S.D. $\pm$  0.054), as contrasted with a mean of 0.218 (S.D. $\pm$  0.058) for 136 females. Although this difference is not great, the probability that it would occur by chance alone is between 1 and 2 in 100.

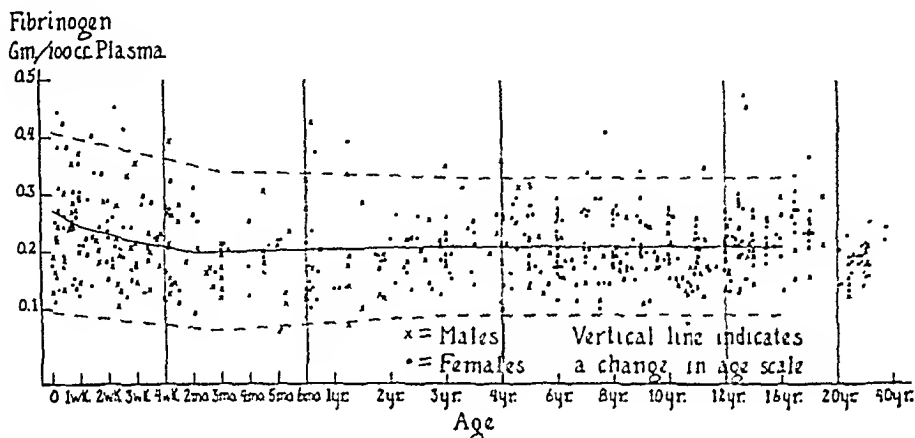


Chart 5.—A scatter diagram of fibrinogen, expressed as grams per 100 c.c. of plasma, referred to age (see legend of Chart 1).

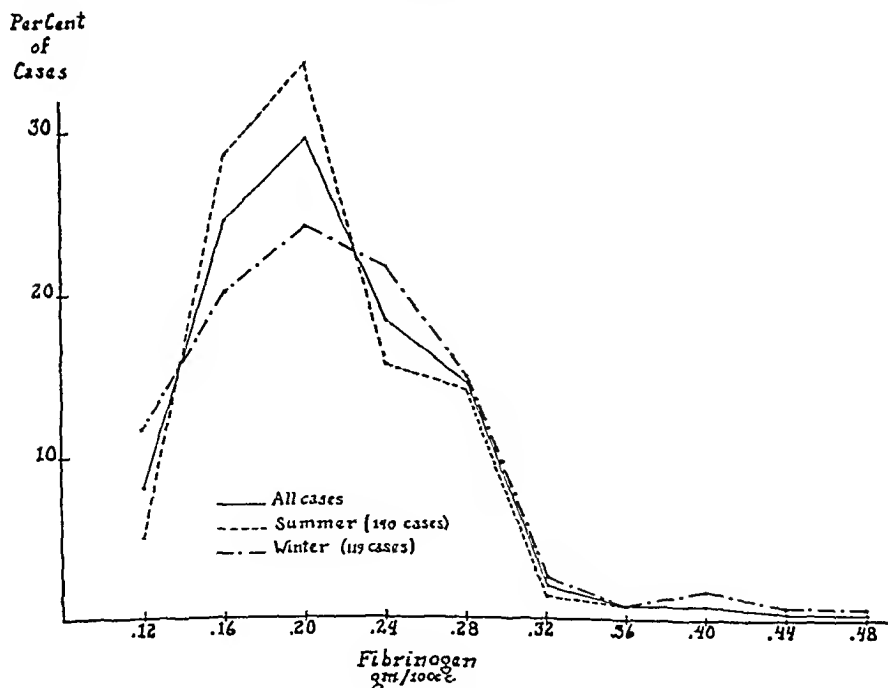


Chart 6.—Frequency polygon of fibrinogen values in 259 analyses on persons over 5 years of age.

The distribution of fibrinogen values about the mean, as shown in Chart 6, shows a skew toward the higher values somewhat similar to that observed with the globulin. This similarity raises a question of correlation between the two

fractions. Chart 7 shows the fibrinogen values in relation to each corresponding globulin value for all analyses on persons over 5 years of age. Although a slight correlation is present, it is to be noted that, with one exception, the fibrinogen values above two standard deviations do not occur in the same persons as those showing globulin values above the two standard deviation range. It seems likely, therefore, that the factor, or factors, producing the skew in the globulin distribution are not the same as those involved in the fibrinogen distribution.

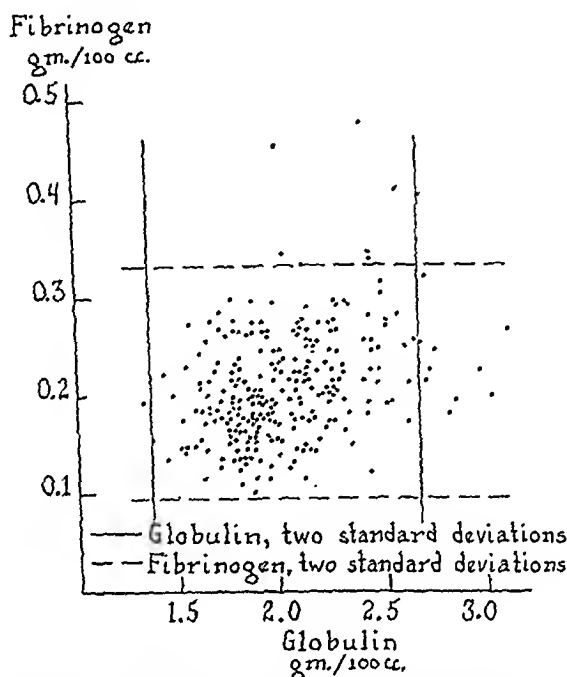


Chart 7.—A scatter diagram of fibrinogen concentration referred to the corresponding globulin concentration in 259 samples of plasma from individuals over 5 years of age.

Ham and Curtis<sup>16</sup> have reviewed the literature with respect to fibrinogen values that have been reported, and have studied this factor in a series of adults under varying physiologic and pathologic conditions. In their discussion these authors state, "Normal pregnancy is the only 'physiological' condition associated with increased fibrinogen concentrations to values of from 350 mg. to 450 mg. Diagnostically, therefore, an abnormally elevated plasma fibrinogen concentration in the absence of pregnancy is laboratory evidence for an organic disease process which may be present at the time of observation or which may have terminated several days or weeks before." Our series shows plasma fibrinogen concentrations above 0.4 Gm. per 100 e.e. in 4 of 259 persons over 5 years of age. At the time of the analyses these persons, as stated previously, had had no minor illness (mild colds) in the preceding two weeks, and no serious illness with fever during the preceding three months. One of the four had recovered from pneumonia three and one-half months before the determination was done and may have had an elevated fibrinogen which would extend over this period of time. The other three children had not shown symptoms of any acute or

chronic disease, nor had they been immunized against any disease during an eight-month period preceding the plasma protein determination. One must conclude, therefore, that (1) the findings of Ham and Curtis do not apply to children; (2) allowance must be made for an occasional "normal" plasma fibrinogen concentration above the limits set by these authors; or that (3) the fibrinogen elevation following disease is more prolonged than these authors would lead one to believe. With the data at hand we have been unable to determine which of these three possibilities is the correct interpretation of our results.

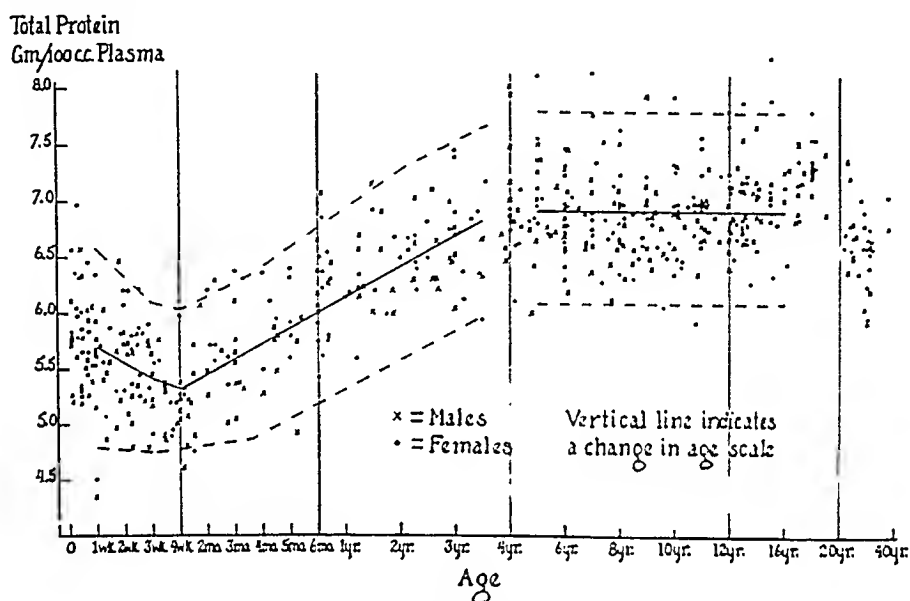


Chart 8.—A scatter diagram of total protein, expressed as grams per 100 c.c. of plasma, referred to age (see legend of Chart 1).

*Total Protein.*—The total protein is frequently determined without separate analysis of the different fractions. This practice leaves much to be desired, since most of the functions of the proteins may be attributed primarily to one or another of the fractions rather than to their combined, total concentration.<sup>17</sup> On the other hand, the determination of total protein is a relatively simple procedure and gives some information of value to the clinician. The total protein reflects the difference between the sexes with respect to fibrinogen and the seasonal variation observed in the albumin; however, the most significant variation is that occurring with age. At birth the total protein concentration is 5.70 Gm. per 100 c.c., with a standard deviation (total) of  $\pm 0.45$  Gm. per 100 c.c. During the first month of life the value falls to 5.33 Gm. per 100 c.c. ( $S.D._T = \pm 0.37$ ), after which there is a gradual rise until the adult level of 6.94 ( $S.D._T = \pm 0.47$ ) is reached at about 4 years of age. These changes are shown graphically in Chart 8.

#### DISCUSSION

Of the many methods used in the study of plasma protein concentrations, the one based upon Kjeldahl analysis has best survived the test of time. This is because the results obtained with this method may be more accurately repro-

duced, and are less subject to errors caused by extraneous factors which may be present, especially in pathologic conditions (see Chorine,<sup>7</sup> and Myers and Muntwyler<sup>20</sup>). In the analysis of fibrinogen the values obtained by different methods may be more comparable. Our discussion of albumin and globulin concentrations will be limited to the studies in which a salting-out procedure has been used to separate the fractions, and the analysis has been based upon Kjeldahl nitrogen determination. In some instances, workers have used a factor other than 6.25 to convert the nitrogen content to protein. These figures have been recalculated using 6.25 as the factor in order that all the results may be expressed in the same terms. We do not wish to imply a belief that the different proteins contain exactly 16 per cent nitrogen; however, this nitrogen content is most uniformly assumed, and the protein concentrations calculated upon this basis may be easily corrected at some later date if it is shown that their nitrogen content varies significantly from this value.

Table II gives a summary of albumin and globulin values reported in the literature. Some of these workers have used serum; others have used plasma. In the latter group both heparin and oxalate have been employed as anti-coagulants, and in two instances no statement is made concerning the anti-coagulant. Of the six workers who have analyzed plasma, only two give values for fibrinogen. In three papers the sum of the mean values for albumin and globulin approximates the mean total protein, and it is, therefore, assumed that the so-called "globulin" includes both globulin and fibrinogen. In the report of Muntwyler, Way, Binns, and Myers,<sup>27</sup> the mean total protein is greater than the sum of the albumin and globulin, and we have, therefore, assumed that the fibrinogen was determined separately. A variety of salts has been used to separate the albumin and globulin. We have not attempted to analyze the reported values on this basis, but we have included all papers in which a salting-out procedure was employed, irrespective of the salt used. In none of the work to be discussed has any mention been made of the season of the year in which the analyses were done. Seasonal variation in the albumin fraction might, to some extent, account for the disagreement among different workers with respect to this fraction.

The data summarized in Table II cover analyses on 102 adults and 199 children from birth to 15 years. For the entire group of adults the mean albumin concentration is about 4.6 Gm. per 100 c.e., with a range from the low of 3.3 reported by Lewinski<sup>21</sup> to a high of 5.65 reported by Bruckman, D'Esopo, and Peters.<sup>26</sup> This mean value is in relatively good agreement with the mean albumin concentration of 4.7 Gm. per 100 c.e., which we have found in older children and adults, and only the low values reported by Lewinski<sup>21</sup> and by Linder, Lundsgaard, and Van Slyke<sup>22</sup> fall outside the three standard deviation range covered by our series. On the other hand, the mean values reported by the different workers for their respective small groups of persons vary widely with respect to each other and in comparison with our mean.

The globulin analyses reported in the literature cover 54 adults, with a combined mean of about 2.0 Gm. per 100 c.e. and a range from 1.32 to 3.79. This mean value is the same as we have found for older children and adults, as is the total range, except for one high value (3.79) reported by Lewinski.<sup>21</sup>

TABLE II

PLASMA PROTEIN VALUES REPORTED IN THE LITERATURE IN WHICH THE METHOD OF ANALYSIS IS SIMILAR TO THAT EMPLOYED IN THIS STUDY

| AUTHOR<br>DATE<br>REFERENCE                                    | NUMBER<br>OF<br>CASES | AGE                       | ALBUMIN†             | GLOBULIN‡            | FIBRINOGEN‡  | REMARKS                                       |
|--|-----------------------|---------------------------|----------------------|----------------------|--|---|
| Lewinski (1903) <sup>21</sup>                                  | 3                     | Adult                     | 3.30-4.47            | 2.36-3.79            | 0.28-0.50  | Oxalated plasma                               |
| Linder, Lunds-<br>gaard, and Van<br>Slyke (1924) <sup>22</sup> | 7                     | Adult                     | 3.62-4.9<br>(4.11)   | 2.45-2.89<br>(2.61)  | In globulin?   | Oxalated plasma                               |
| Salvesen (1926) <sup>23</sup>                                  | 16<br>males           | Adult                     | 3.95-5.24<br>(4.44)  | 1.96-3.16<br>(2.58)  | In globulin?   | Oxalated plasma                               |
|  | 16<br>females         | Adult                     | 3.77-4.80<br>(4.55)  | 2.18-3.55<br>(2.68)  | In globulin?   | Oxalated plasma                               |
| Wiener and<br>Wiener (1930) <sup>24</sup>                      | 20                    | Adult                     | 4.20-5.00<br>(4.60)  | 1.50-1.90<br>(1.70)  | Male<br>0.22-0.28<br>(0.25)<br><br>Female<br>0.25-0.33<br>(0.28) | Anticoagulant<br>not given<br><br>Plasma used |
| Moore and Van<br>Slyke (1930) <sup>25</sup>                    | 9                     | Adult                     | 4.0-4.5<br>(4.3)     | (2.8)                | In globulin?   | Heparinized plasma                            |
| Bruckman,<br>D'Esopo, and<br>Peters (1930) <sup>26</sup>       | 13<br>males           | Adult                     | 4.37-5.65<br>(5.06)  | 1.32-2.91<br>(1.89)  | -----  | Serum   |
|  | 8<br>females          | Adult                     | 4.71-5.17<br>(4.98)  | 2.02-3.22<br>(2.62)  | -----  | Serum   |
| Muntwyler, Way,<br>Binns, and<br>Myers (1933) <sup>27</sup>    | 10<br>males           | Adult                     | (4.84)               | (1.88)               | ?  | Anticoagulant<br>not given<br>Plasma used     |
| Darrow and Cary<br>(1933) <sup>28</sup>                        | 20                    | Newborn<br>over 3<br>days | (3.73)<br>S.D. -0.38 | (1.78)<br>S.D. -0.45 | -----  | Serum   |
|  | 16                    | 5-6 mo.                   | (4.28)<br>S.D. -0.38 | (2.01)<br>S.D. -0.34 | -----  | Serum   |
| Rennie (1934) <sup>29</sup>                                    | 6                     | 3-6 mo.                   | (4.67)               | (1.84)               | -----  | Serum   |
|  | 4                     | ½-1 yr.                   | (4.76)               | (1.99)               | -----  |   |
|  | 5                     | 1-1½ yr.                  | (5.26)               | (2.27)               | -----  |   |
|  | 20                    | Over 1½ yr.               | (5.02)               | (2.39)               | -----  |   |
| Rennie (1935) <sup>30</sup>                                    | 22                    | 3-23 mo.                  | 4.1-5.9              | 1.13-2.82            | -----  | Serum   |
|  | 24                    | 2½-11 yr.                 | 4.0-5.5              | 1.4-3.0              | -----  |   |
| Dodd and Minot<br>(1936) <sup>31</sup>                         | 16                    | Birth to 3<br>mo.         | 2.6-4.2<br>(3.66)    | (1.78)               | -----  | Serum   |
|  | 34                    | 3 mo.-2 yr.               | 3.6-4.8<br>(4.28)    | (1.91)               | -----  |   |
|  | 32                    | 2-15 yr.                  | 3.9-5.2<br>(4.65)    | (2.38)               | -----  |   |

\*Patients have "no apparent abnormality except mild rickets."

†Patients are afebrile convalescents.

‡Numbers in parentheses indicate mean values.

In contrast to this, the mean value of 2.6 Gm. per 100 c.c. for globulin plus fibrinogen, reported in the literature for 48 adults, is distinctly above the sum of our mean values for the two fractions.

Serum analyses have been reported in the literature for 199 children. These results have not been presented for each individual, but for age groups which frequently include periods where we have found the concentration of one of the



fractions to be changing. Thus the mean values for these groups cannot be exactly compared with our series, since we do not know the distribution of persons within the groups that have been reported. In general, the albumin concentrations which have been reported fall well within the range of values covered by our series for corresponding age periods. A notable exception to this is the high mean albumin level of 5.07 Gm. per 100 c.c. reported by Rennie<sup>29</sup> in 1934 for 25 children over 1 year old. This is in contrast to our mean of 4.7 for all children over 1 year old, or the mean of 4.83 for the analyses done during the winter months on children of this age group.

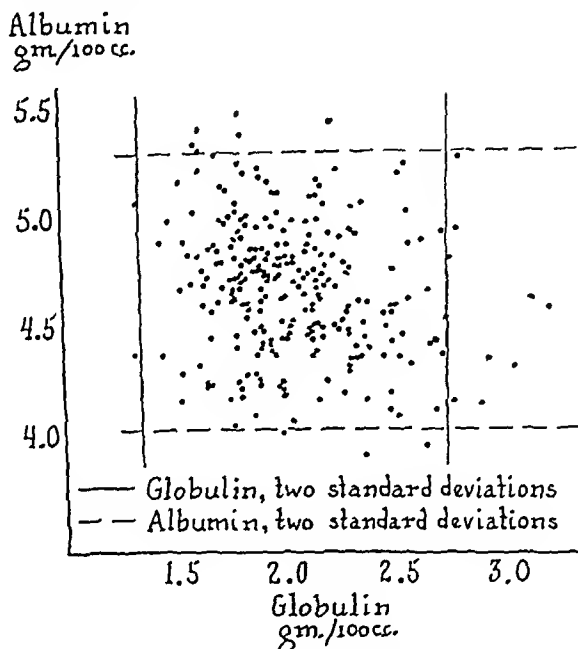


Chart 3.—A scatter diagram of albumin concentration referred to the corresponding globulin concentration in 262 samples of plasma from individuals over 5 years of age.

The serum globulin values which have been reported for children tend to be higher than those of our series at corresponding age levels. A possible explanation of this discrepancy might be that some of the children used by other workers, although not acutely ill when the blood was taken, may have shown a mild hyperglobulinemia as a result of recent infection or immunization.

Ham and Curtis<sup>10</sup> have reviewed the literature with respect to plasma fibrinogen concentration in normal persons. In 193 determinations on adults reported by these authors, and in the work reviewed by them, the fibrinogen values fall between 0.190 and 0.380 Gm. per 100 c.c., with a mean of about 0.250. These results are essentially the same as those given in Table II, as reported by Lewinski,<sup>21</sup> and by Wiener and Wiener.<sup>24</sup> Our mean value for persons between 3 and 20 years is slightly below this, and our range is somewhat wider, including many values below those found in the literature, and a few which are higher. We have been unable to find any extensive literature covering the plasma fibrinogen concentration in early childhood or infancy. It is

quite possible that our results on persons from 3 to 20 years of age would not represent the values to be expected in persons from 16 to 61 years, the age range studied by other workers. In our series there is an apparent rise in the fibrinogen in the period from 12 to 20 years. This is offset by the low values observed between 20 and 40 years, and the mean for the entire group beyond 12 years is the same as that for children from 3 to 12 years of age. A real tendency toward increasing fibrinogen concentration after puberty can be established only by studies on a larger group of persons. The results reported in the literature may indicate that such an increase does occur.

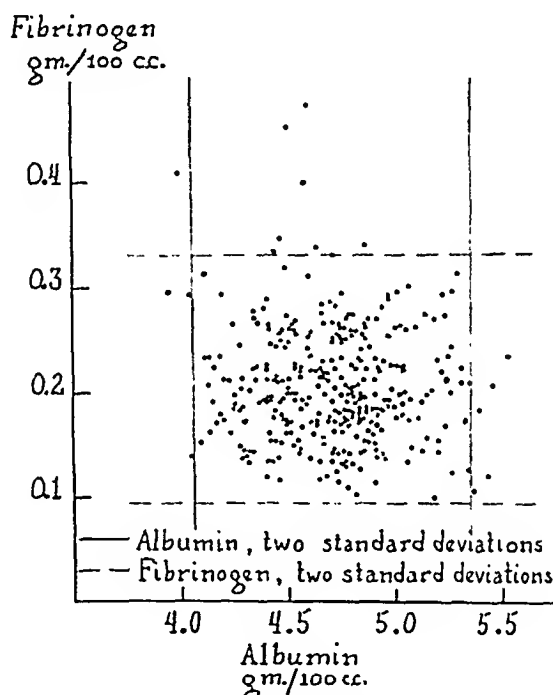


Chart 10.—A scatter diagram of fibrinogen concentration referred to the corresponding albumin concentration in 259 samples of plasma from individuals over 5 years of age.

We are not satisfied that the ratios of albumin to globulin have sufficient significance to justify including these values in our tables. It is true that plasma and serum protein determinations are frequently reported in terms of the total protein concentration and the ratio of albumin to globulin, the A./G. ratio. With serum analysis this method of recording, although unnecessarily obscure, gives a complete picture, for one can calculate the more significant individual values for albumin and globulin if the total protein and the A./G. ratio are given. When plasma is used, however, confusion arises because some workers include the fibrinogen with the globulin in computing the A./G. ratio, while others do not.

Interpretation of the results of plasma protein determinations, from either the physiological or clinical point of view, is based largely upon the concentration of each separate fraction, with its independent functions, and not upon the ratios between the different parts. Furthermore, in our work with healthy persons, the three major protein fractions have been found to vary independently

of each other, as shown in Charts 7, 9, and 10. Therefore, we believe that the albumin, globulin, and fibrinogen should be treated as separate entities (or systems), and that it is more satisfactory to record in the literature the actual values of the separate protein fractions rather than a ratio between any of the constituent parts.

Boissevain<sup>18, 19</sup> has recently reported that serum albumin greatly promotes, and serum globulin inhibits, the growth of the tubercle bacillus on artificial media. Should the two proteins be shown to have an antagonistic action in tuberculosis, the A./G. ratio would become of importance in so far as these patients are concerned.

#### SUMMARY AND CONCLUSIONS

1. A total of 566 analyses of plasma albumin, globulin, and fibrinogen have been made on 547 healthy persons from birth to 39 years of age.

2. The protein fractions have been separated by salting-out with sodium sulfate and analyzed by a micro-Kjeldahl procedure.

3. Each of the protein fractions undergoes a change with age in early infancy and childhood. These changes have been defined.

4. The plasma albumin concentration in persons over 3 years of age is higher in the winter than in the summer months. This seasonal variation does not appear in the other fractions.

5. In persons over 5 years of age the plasma fibrinogen tends to be higher in females than in males.

6. In normal persons the albumin, globulin, and fibrinogen vary independently.

7. No correlation has been found between any of the protein concentrations and the time elapsing after the ingestion of food.

8. There is no correlation between any of the protein concentrations and the height, weight, or body surface of the individuals.

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# BLOOD FROM THE EAR LOBE\*

## PRELIMINARY REPORT

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IT SEEMS generally accepted that, under standard conditions, practically identical hemoglobin values are obtained from venous and peripheral blood. A closer review of the literature, however, reveals that this view is based in most cases only on comparison of venous blood with blood from the finger tip. Only a few investigators have worked with blood from the ear lobe as representing the peripheral sample, and have found no significant differences for hemoglobin or red blood cells (Bürker,<sup>1</sup> Yarbrough,<sup>2</sup> Foord,<sup>3</sup> Reichel and Monastero,<sup>4</sup> Price-Jones and co-workers<sup>5</sup>).

During hematologic work at this laboratory concerned with the establishment of standard red blood cell values for Palestine and the study of seasonal influences on these values, the usually taken venous samples were—very occasionally—replaced by samples from the ear lobe; in each of these cases, abnormally high hemoglobin figures were observed. A reinvestigation of the question seemed, therefore, advisable, in spite of the fact that it appears to be settled.

### EXPERIMENTAL

As mentioned this study was undertaken as a corollary to standard work, which has been carried out along the internationally adopted lines of such investigations and will be described in detail in a forthcoming publication. If not otherwise stated, the subjects comprised healthy adults between 20 and 40 years of age, presenting themselves before breakfast or four hours after a meal. Blood was taken from the cubital arm vein without tourniquet, Liquoid Roehet being used as the anticoagulant, which, like heparin, causes no shrinkage during the hematocrit determinations.

After wiping the finger pulp and the ear lobe with a little ether, the peripheral samples were simultaneously taken by puncture with a spring lancet, care being taken to avoid squeezing or cyanosis. If only hemoglobin was determined, no anticoagulant was used; otherwise approximately 0.5 c.c. of peripheral blood was collected in small vials prepared with the anticoagulant, as described by Andresen.<sup>6</sup>

Hemoglobin was determined spectrophotometrically after reduction with sodium hydrosulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) according to Heilmeyer,<sup>7</sup> using a Leitz extinction photometer with screen 570  $\mu$ . This method, which has also been used by Bürker<sup>1</sup> and Williamson,<sup>8</sup> has proved, after standardization, very satisfactory in our hands. To demonstrate the accuracy of the method, the results of 10 independent determinations on the same blood sample are given:

\*From the Chemical Laboratory, Hadassah Municipal Hospital, Tel Aviv.

†Sodium polyanethole sulfonate.

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TABLE I

SIMULTANEOUS HEMOGLOBIN DETERMINATIONS ON VENOUS AND PERIPHERAL BLOOD  
(Results in Grams Per Cent)

| NO. | NAME | VENOUS | FINGER TIP | EAR LOBE | REMARKS                       |
|-----|------|--------|------------|----------|-------------------------------|
| 1   | Lau  | 14.8   | 14.3       | 15.9     | Normocytic anemia (pregnant)  |
| 2   | Bru  | 15.2   | 15.2       | 16.1     |                               |
| 3   | Gla  | 15.8   | 15.8       | 17.0     |                               |
| 4   | Wal  | 15.4   | 15.4       | 19.3     |                               |
| 5   | Kov  | 16.4   | 15.7       | 17.5     |                               |
| 6   | Dre  | 15.0   | 15.4       | 16.3     |                               |
| 7   | Fis  | 15.2   | 15.2       | 18.3     |                               |
| 8   | Loc  | 11.3   | 11.3       | 12.0     |                               |
| 9   | Mel  | 15.7   | 16.1       | 16.3     |                               |
| 10  | Gru  | 13.4   | 13.2       | 16.1     |                               |
| 11  | Fei  | 14.5   | 13.6       | 14.5     | Macrocytic anemia             |
| 12  | Ste  | 13.4   | 13.4       | 17.5     |                               |
| 13  | Lae  | 15.2   | 16.2       | 14.8     |                               |
| 14  | Jac  | 15.4   | 14.3       | 15.4     |                               |
| 15  | Bru  | 13.8   | 12.5       | 14.0     |                               |
| 16  | Ber  | 13.8   | 13.4       | 15.2     |                               |
| 17  | Wei  | 16.6   | 16.6       | 17.7     |                               |
| 18  | Fis  | 14.2   | 13.6       | 15.6     |                               |
| 19  | Mar  | 15.7   | 14.0       | 17.0     |                               |
| 20  | Kar  | 12.5   | 11.8       | 14.0     |                               |
| 21  | Jac  | 14.5   |            | 14.7     | Microcytic hypochromic anemia |
| 22  | Kop  | 10.5   |            | 10.8     |                               |
| 23  | Dre  | 16.7   |            | 17.2     |                               |
| 24  | Lau  | 11.8   |            | 14.0     |                               |
| 25  | Bru  | 12.7   |            | 12.9     |                               |
| 26  | Mey  | 9.6    |            | 11.4     |                               |
| 27  | Kor  | 12.5   |            | 12.5     |                               |
| 28  | Mac  | 13.4   |            | 16.3     |                               |
| 29  | Mey  | 13.4   |            | 15.7     |                               |
| 30  | Rem  | 13.1   |            | 14.7     |                               |
| 31  | Bru  | 15.1   |            | 16.1     | Microcytic hypochromic anemia |
| 32  | Fis  | 14.2   |            | 15.6     |                               |
| 33  | Gur  | 14.4   |            | 15.0     |                               |
| 34  | Mos  | 17.6   |            | 18.4     |                               |
| 35  | Mir  | 13.2   |            | 15.4     |                               |
| 36  | Bin  | 13.6   |            | 14.5     |                               |
| 37  | Gef  | 15.0   |            | 17.7     |                               |
| 38  | Sch  | 7.5    |            | 8.8      |                               |
| 39  | Tra  | 14.0   |            | 15.9     |                               |
| 40  | Lev  | 15.7   |            | 17.0     |                               |

Mean value: 15.96 grams per cent. Standard deviation: 0.44. Standard error: 0.14. Coefficient of variation: 2.78.

## RESULTS

Table I gives the results of the simultaneous determinations on venous and peripheral blood, obtained throughout the year. Ten additional comparisons of venous and finger tip blood are for the sake of brevity not listed in the table, but are included in the statistical evaluation given in Table II.

TABLE II

STATISTICAL EVALUATION OF 30 COMPARISONS OF VENOUS AND FINGER-TIP BLOOD

| SOURCE     | MEAN VALUE | STANDARD DEVIATION | STANDARD ERROR | COEFFICIENT VARIATION | DIFFERENCE OF MEANS | STANDARD ERROR OF DIFFERENCE |
|------------|------------|--------------------|----------------|-----------------------|---------------------|------------------------------|
| Venous     | 14.40      | 2.00               | 0.365          | 13.9                  | -0.25               | 0.52                         |
| Finger tip | 14.15      | 2.06               | 0.376          | 14.6                  |                     |                              |

Table II also shows the evaluation of 30 simultaneous measurements of venous and finger-tip blood, obtained from 27 persons (14 males, 13 females), 25 of them healthy ones. The difference of the mean values of  $-0.25$  Gm. is by far too small to be statistically significant. This would be the case only if it were more than twice its standard error (Fisher<sup>9</sup>). Ten of the 30 cases gave identical values; in 5 cases the finger-tip values were higher; in 15 cases they were lower than the venous ones.

TABLE III

STATISTICAL EVALUATION OF 40 COMPARISONS OF VENOUS AND EAR LOBE BLOOD

| SOURCE   | MEAN<br>VALUE | STANDARD<br>DEVIATION | STANDARD<br>ERROR | COEFFICIENT<br>VARIATION | DIFFERENCE<br>OF MEANS | STANDARD ERROR<br>OF DIFFERENCE |
|----------|---------------|-----------------------|-------------------|--------------------------|------------------------|---------------------------------|
| Venous   | 14.03         | 1.96                  | 0.310             | 14.3                     |                        |                                 |
| Ear lobe | 15.38         | 2.14                  | 0.338             | 13.9                     | +1.35                  | 0.46                            |

Table III shows the evaluation of 40 simultaneous measurements of venous and ear lobe blood. These were carried out on 34 persons (12 males, 22 females), 4 of them suffering from anemia. The difference of mean values,  $+1.35$  Gm., is nearly three times as great as its standard error and must, therefore, be considered statistically significant. In 3 cases both values were identical; in one case the ear lobe contained less, in 36 cases, it contained more hemoglobin than the arm vein. On the average, the ear lobe values are 10 per cent higher than the venous ones.

It should be emphasized that the manner in which the blood was taken from the ear lobe seemed to be without influence, as could be observed in separate experiments. The same high values were obtained whether a deep puncture wound with consequent free flow was made, or whether the blood was obtained by repeated squeezing. No change in the picture was seen when the ear blood was taken first, and the venous sample after a few minutes interval. Seasonal influences seemed likewise without effect. More than half of the trials were carried out during the winter and spring season, whose climatic conditions do not show very marked difference from those of American and European countries of the moderate zone.

## COMMENT

Differences between venous and peripheral blood under standard conditions have so far been reported only in the case of pernicious anemia by Duke and Stofer,<sup>10</sup> who found higher values in the ear lobe. During the first days or weeks of life higher hemoglobin, red blood cell, and cell volume values were likewise observed by several authors in peripheral blood from the heel (e.g., Andresen and Mugaage<sup>11</sup>). The notorious high peripheral values in shock, caused by blood concentration, need scarcely be mentioned here.

The fact that such differences also exist in the healthy (and anemic!) adult in the case of the ear lobe should discourage the practice of taking blood from this source for hematologic diagnosis, a practice which is still used by a considerable number of physicians throughout the world. It further offers a possible explanation for the fact that the hemoglobin values in Williamson's<sup>8</sup>

classic investigation are uniformly about 10 per cent higher than those obtained by the majority of all other workers. Williamson had been working with blood from the ear lobe.

It seems conceivable that stasis in the capillaries and subsequent changes in the blood composition may more easily occur in the ear lobe than in the finger pulp. For a better understanding of the phenomenon, however, it is imperative to add red blood cell and hematocrit determinations to the hemoglobin values reported in the present paper. Work on this line is now in progress, and it can already be stated that a tendency for macrocytosis prevails in the ear lobe blood. It would be worth while to investigate if this also applies to body blood deposits.

#### SUMMARY

Venous blood has been compared under standard conditions with peripheral blood from the finger tip and the ear lobe.

No significant differences exist between the hemoglobin content of venous and finger-tip blood. Blood from the ear lobe, however, contains significantly more hemoglobin and should, therefore, not be used for diagnostic purposes.

I wish to express my gratitude to Dr. I. L. Fisher for his helpful advice and criticism throughout this study.

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# THE MEASUREMENT OF INTRAVENOUS BLOOD SUGAR CURVES\*

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THE blood sugar curve, obtained after the administration of glucose, is a clinical laboratory test frequently employed, but only rarely giving any clear-cut information, except in cases of markedly abnormal carbohydrate metabolism. Although it is well known that there are great normal variations in blood sugar curves, variations noted when examining a number of persons, as well as one and the same person on different occasions, there is no simple way of describing these variations.

The following report describes a method by which the intravenous blood sugar curve may be characterized by a single figure. The method is based on the fact that, although a curve is difficult to describe, it is easy to measure the slope of a straight line. Many curves may be considerably straightened out by plotting on logarithmic paper; often this is true of the intravenous blood sugar curve to a very surprising degree, especially in the middle part of the curve, where the values fall in the range of normal postabsorptive values (from about 200 to 100 mg. per 100 c.c.). This is, probably, the most important part of the curve. In the higher ranges, never reached under normal conditions, it is possible that special mechanisms come into play (for instance, excretion of sugar through the kidneys). Toward the end of the curve, at values of about 100 mg. per 100 c.c., we might expect various mechanisms for stabilizing the blood sugar at normal values to become active. In the middle range, however, we would observe and study the normal mechanism for removal of excess carbohydrate from the blood after ingestion of carbohydrate, and it is interesting that it is in this range that the logarithmic curve so often forms a straight line.

## METHOD

The blood sugar values are plotted on semilogarithmic graph paper, and the part which forms an approximately straight line is noted. In questionable cases, where it is difficult to decide how much of the curve is approximately straight, the decision is not of very great importance, as long as the selected part includes most of the values between 200 and 100 mg. per 100 c.c. As will be shown later, including one value more or less will not greatly change the final result. The logarithms of the blood sugar values forming the end points of the selected part of the curve are then obtained, with two decimals, from a table or slide

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rule. The difference between these two logarithms, divided by the time elapsed between these two points of the curve, is an approximate measure of the slope of the curve.

TABLE I

| MIN. AFTER INJECTION | BLOOD SUGAR | LOG BLOOD SUGAR | CHANGE IN BLOOD SUGAR |
|----------------------|-------------|-----------------|-----------------------|
| 2                    | 280         | 2.45            |                       |
| 15                   | 220         |                 | 60                    |
| 30                   | 161         |                 | 51                    |
| 45                   | 124         |                 | 37                    |
| 60                   | 99          | 2.00            | 25                    |
| 75                   | 101         |                 | +2                    |
| 90                   | 98          |                 | 3                     |

Table I gives the blood sugar values obtained in Case 2 (third from the left in the second row in Fig. 1). An approximately straight line is formed between the values 280 (first value) and 99 (fifth value). The difference between the logarithms of these two values is  $2.45 - 2.00 = 0.45$ . The value 280 was obtained two minutes after the injection of glucose; the value 99, sixty minutes after the injection; the elapsed time is, therefore, 58 minutes. In this period of time the logarithm of the blood sugar decreased 0.45. In one minute, then, it decreased on the average 0.00776. This value, 0.00776 per minute, is a measurement of the slope of the logarithmic curve. However, a decrease of the logarithm of the blood sugar of 0.00776 per minute is the same as if the actual blood sugar value had been divided by 1.0181 each minute. Dividing by 1.0181 is the same as multiplying by 0.9823. In other words, the blood sugar at the end of each minute is in this case on the average 98.23 per cent of what it was at the beginning of the minute, which is a decrease of about 1.77 per cent. This figure, 1.77 per cent per minute, is the measure of the rate of decrease of sugar in the blood in this case. Fortunately, this rather complicated calculation made to arrive at this figure is not necessary. If the slope of the logarithmic curve (in this case 0.00776) is multiplied by 230, we will always arrive at approximately the same result as that obtained in the above calculation.\* In this case, we get 1.78 per cent per minute by using this method, while the more elaborate calculation resulted in the value 1.77 per cent per minute. As the accuracy of the method justifies the inclusion of only one decimal, the difference is not important. For practical purposes it is sufficient to say that the rate of decrease of blood sugar in Case 2 was 1.8 per cent per minute.

*It must be very strongly emphasized that this figure, 1.8 per cent per minute, is not the same as 18 per cent in ten minutes, or 180 per cent in one hundred minutes; that is, it is not the same if percentage change is calculated, as is usually done, in per cent of the initial figure at the beginning of the period of change. If, however, the percentage change is calculated in per cent of the geometric mean between the initial and the final figure during the period of change, then it will be approximately correct to multiply per cent per minute by any number of minutes.*

\*This statement is based on the fact that  $\frac{d}{dt}(\log_e y) = \frac{1}{y} \cdot \frac{dy}{dt}$ .

The multiplication by 100 is made to change a fraction into percentage; by 2.30, to change ordinary into natural logarithms.

*Summary of Method.*—Plot the blood sugar curve on semilogarithmic paper (obtainable in most stationery stores). Determine which part of the curve is approximately straight. Obtain from a table or slide rule the logarithms (with two decimals) of the two figures forming the beginning and end of the straight part of the curve. Take the difference between these two logarithms, divide this difference by the number of minutes elapsed between the two blood sugar determinations, and multiply the result by 230. The figure obtained is the average rate of decrease of the blood sugar in per cent per minute.

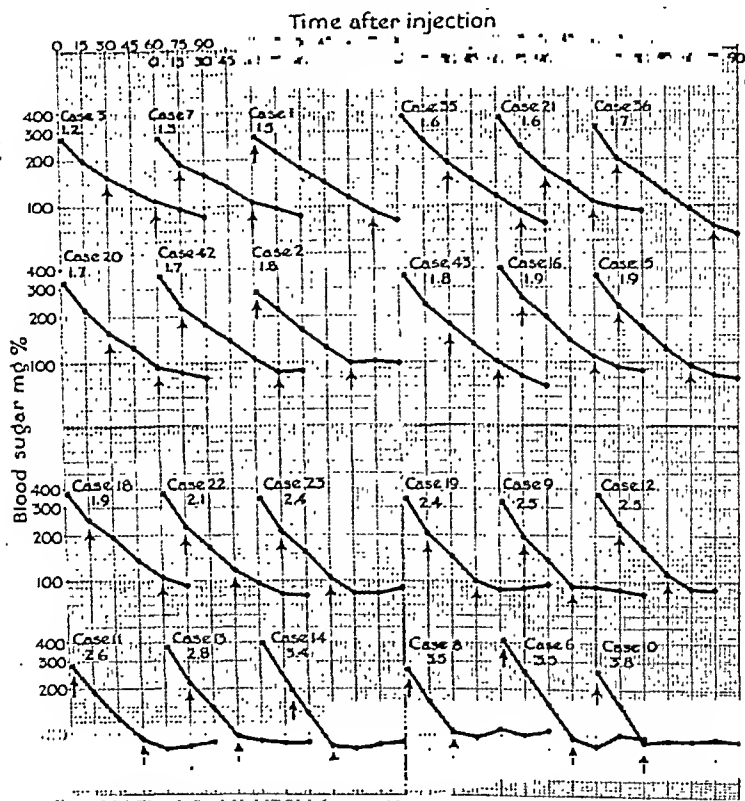


Fig. 1.—Intravenous blood sugar curves from approximately normal persons plotted on logarithmic paper. The part of the curve used for estimating the slope is marked by arrows. The figures indicate the slope expressed in per cent per minute decrease.

We have made intravenous blood sugar curves on a number of patients with miscellaneous disorders, all children; old burns, kept in the hospital for grafting; patients with gonorrheal vaginitis; fractures; patients with bronchiectasis. Some of these have received a diet high in carbohydrate, others a diet low in carbohydrate; many were tested on both diets. We shall not give the data as to the composition of the diet, since the lack of a metabolism ward made it quite certain that the diets were only approximately followed. The diets as offered had a ketogenic-antiketogenic ratio of about 0.25:1 for the high carbohydrate diet, 2:1 for the low carbohydrate diet. All we can definitely say is that these two diets were very different in their carbohydrate content.

The technique of the intravenous test is important for the interpretation of the curve. It goes without saying that the amounts of sugar given should be

strictly standardized. In our tests we have given 0.5 Gm. of glucose per kilogram of body weight in a 20 per cent solution (in normal saline). The amounts of dextrose injected have a marked effect on the curve.<sup>1</sup> In some cases we have given smaller amounts and found that on these smaller amounts the curves tend to be irregular and difficult to interpret. The time used for injection should be made as uniform as possible by using the same size intravenous needle and the same pressure in every case. We have used a No. 18 intravenous needle and a salvarsan tube with very rapid drip at a distance of six feet from the floor. In this manner, the injection was usually completed in less than one minute. According to Tunbridge and Allibone,<sup>1</sup> small differences of two to four minutes in the time used for injection are of no importance for the result.

### RESULTS

In Fig. 1 are plotted on logarithmic paper our 24 patients, more or less normal, on a high carbohydrate diet. The curves are ranged according to the steepness of the slope, and the figures above the curves indicate the steepness in terms of per cent decrease per minute. The part of each curve chosen for the calculation of the slope is indicated by the arrows. It may seem as if this part, in some cases, was rather arbitrarily chosen, but as has already been pointed out, it makes very little difference in the result of the calculation if one only adheres to the principle of (1) choosing as straight a part of the curve as possible, and (2) in questionable cases to include approximately the range from 200 to 100 mg. per cent. In Case 20, for instance, it may seem questionable whether to calculate from the second or the third value; calculating from the second, one obtains a slope of 1.9 per cent per minute; calculating from the third, the value obtained is 1.7 per cent per minute. In Case 3, calculating from the second value the slope is 1.4, from the third value, it is 1.2 per cent per minute. Going over a number of curves in this manner we have found that the error of the method is, approximately, 0.2 per cent, as far as the calculation of the slope is concerned. It seems likely that if we assume a total error of twice as much,  $\pm 0.2$  per cent, we shall not overestimate the accuracy of the method. That would mean that values more than 0.4 per cent apart should be considered as showing a real difference. The total range of our values is from 0.9 to 3.8 per cent per minute (a difference of 2.9 between the highest and the lowest value), and with an error of 0.4 per cent we would, then, be justified in recognizing seven different degrees of steepness. This, it seems to us, is a worth-while refinement on the usual method of designating the curves as "steep," "normal," or "flat."

In Table II may be seen the distribution of the values for the slope of the curve (the removal rate of sugar from the blood) in patients on a high and on a low carbohydrate diet. (Four more curves are included than in Fig. 1, the additional four being repeated curves on cases previously tested.) The table shows how on the high carbohydrate diet the most common removal rate was between 1.5 and 1.9 per cent per minute (10 cases), while on the low carbohydrate diet the most common rate was between 1.0 and 1.4 per cent per minute. A very interesting fact stands out: There was considerably more variation

between the cases on a high carbohydrate diet than between those receiving a low carbohydrate intake (16 cases were tested on both diets; the difference in variability may, therefore, hardly be ascribed to a difference in case material). This is all the more striking, since we feel sure that the low carbohydrate diet was more often broken than the high carbohydrate diet. The findings seem to indicate that when the carbohydrate metabolism is understimulated (low carbohydrate diet), the reaction to a sudden demand on this metabolism (intravenous injection of glucose) is very much the same in all persons; while there seems to be considerable individual variation in the response when the metabolism has previously been stimulated by a high carbohydrate intake. We may also conclude that if we plan to study individual variations in carbohydrate metabolism, it seems more likely that a high carbohydrate intake will bring out such variations than a low carbohydrate diet. In some persons, tested on both diets, the difference in response was very marked; in others the difference was small. The difference in response was nearly always due to differences in the response to the high carbohydrate diet, since the removal rate on the low carbohydrate diet was very much the same in all cases.

TABLE II

DISTRIBUTION OF PER CENT PER MINUTE DECREASE OF BLOOD SUGAR AFTER INTRAVENOUS INJECTION

| CARBOHYDRATE DIET | NUMBER OF CASES |         |         |         |         |      |
|-------------------|-----------------|---------|---------|---------|---------|------|
|                   | LESS THAN 1.0   | 1.0-1.4 | 1.5-1.9 | 2.0-2.4 | 2.5-2.9 | 3.0+ |
| High              | -               | 4       | 10      | 5       | 5       | 4    |
| Low               | 1               | 17      | 7       | -       | -       | -    |

TABLE III

| CASE NO. | REMOVAL RATE AFTER 3 DAYS ON HIGH CARBOHYDRATE DIET; % PER MIN. | REMOVAL RATE AFTER DIET CHANGED TO LOW CARBOHYDRATE |              |
|----------|---|---|--------------|
|          |   | AFTER 2 DAYS  | AFTER 5 DAYS |
| 44       | 1.7   | 1.0   | 1.0          |
| 45       | 2.5   | 1.4*  | 1.0          |
| 46       | 1.6   | 1.0   | 1.2          |
| 47       | 2.5   | 1.8   | 1.2          |

\*Technique not quite good.

It is, of course, a well-known fact that the preceding diet is of great importance for the outcome of the blood sugar curve. According to Tunbridge and Allibone,<sup>1</sup> the patient should be on the diet two weeks before the test is made, a condition hardly possible to fulfill in clinical practice. To judge from our findings, the diet is of more importance in some persons than in others. Not only, as mentioned above, that some persons respond very strongly to a high carbohydrate diet (steep curves) while in others the response is much less marked; but it seems also that the maximum effect of a change in diet is obtained sooner in some persons than in others. In Table III may be seen the result obtained by suddenly changing the diet in four cases. In those two (Case 44 and Case 46) where the removal rate was about average on a high carbohydrate diet, no more effect of the change was seen after five days than

was already present after two days. In the two remaining cases, however, the removal rate was rather rapid on the high carbohydrate diet, and in these two cases the effect of this diet seemed to last longer. If the values obtained after only two days on a low carbohydrate diet had been accepted as final, one would, obviously, have obtained an erroneous impression as to the speed of removal on this diet. For this reason, we have included in our table only the cases where the diet had been given at least four days previous to the test.

#### COMMENT

A simple way of measuring the intravenous curve is that used by Crawford,<sup>2</sup> who classifies the curve according to the time it takes after the injection for the blood to reach normal levels. We are of the opinion that the intravenous blood sugar curve deserves a somewhat more accurate method of measuring than is obtained in that manner. For instance, measured according to Crawford, the five first curves in Fig. 1 would be all the same, while a brief look shows that the fifth (Case 21) is steeper than the first (Case 3). The method also would indicate differences where, in our opinion, none exist, as for instance between the fifth case in the first row (Case 21) and the sixth case (Case 36). The former (Case 21) reached the level of 100 mg. in about sixty-seven minutes; the latter (Case 36), in about fifty-five minutes. According to our calculation, the curves are about alike and the difference obtained by the other method of estimation is due entirely to the fact that the curve of Case 21 starts at a higher level than that of Case 36. Compare also the two last curves in the diagram, Cases 6 and 10. Here again, the curves would be very different according to Crawford, but about the same according to our method of measuring. It seems questionable to us whether the absolute height to which the curve rises—which plays such an important role in the method of Crawford—is not much less important than the steepness of the slope, which is the only characteristic measured by our method. In any case, the steepness should be measured; then, as a second characteristic, may, perhaps, be added the maximum rise.

Ross and Tonks<sup>3</sup> are, in our opinion, using a somewhat too complicated method for measuring the intravenous curves. These authors measure the surface area under the curve by means of a planimeter. In addition to this being a somewhat elaborate method, it also seems to us that from a mathematical standpoint the differential of the blood sugar curve, which is determined by our method, has more meaning than the integral, determined by Ross and Tonks.

Although the purpose of our study was to find a practical method of measuring variations in the intravenous blood sugar curve, a fact of more theoretical interest became apparent from our data: Whenever the intravenous curve forms a straight logarithmic line, the rate at which the sugar is being removed from the blood at a given moment is directly related to the amount of sugar present in the blood at that moment; being always the same percentage of the amount of sugar present.

Another interesting fact is that in at least ten of our curves an approximately straight line slope changes suddenly into a horizontal line (L-shaped curves). This indicates that at a given level of blood sugar the removal of sugar from the blood suddenly stops, or, which is more probable, at a given

level sugar begins to enter the blood at the same rate as it is removed. If the latter is true, we would, then, have a method of calculating the rate at which—in a given individual—the sugar enters the blood at a given time; in other words, the rate of flow of sugar from stores and alimentary tract. For instance, if a curve with a slope of 2 per cent per minute suddenly becomes horizontal at the level of 100 mg. per cent, we might assume that this equilibrium is accomplished by an inflow of 2 per cent per minute, or approximately 2 mg. for every 100 c.c. of blood. If such a person had 10 liters of blood, he would have to consume, on the average, 12 Gm. of sugar per hour to maintain the rate of supply of sugar to the blood. It may thus be seen that the assumption we have made leads to rather reasonable figures for daily carbohydrate intake.

#### SUMMARY

A method is described by which the slope of the intravenous blood sugar curve may be calculated in a simple manner and described by a single figure.

By using this method, it was shown that the slope of the intravenous curves was surprisingly constant when the patients received a low carbohydrate diet; on a high carbohydrate diet the patients showed considerable individual variations.

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## THE USE OF DIHYDROTACHYSTEROL IN PARATHYROIDIC TETANY\*

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### REPORT OF A CASE

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THE occurrence of tetany following removal of the thyroid gland with destruction or loss of the parathyroids is unusual, owing to the improvement in surgical technique that has taken place. There are normally four parathyroid glands, two on each side of the thyroid, but their number and location vary considerably due to defects in embryologic development. Because of this anomaly and their small size, they are often partly removed. Hypertrophy of the remaining glandular tissue usually brings about a cure in a few months. In rare instances so much gland tissue has been removed that tetany becomes a permanent condition. The case reported here is of this type, since regeneration did not occur after one and a half years, and the treatment presented an ideal clinical subject for observation of the effect of a new drug.

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Dihydrotachysterol is made from tachysterol, a by-product of the irradiation of ergosterol with ultraviolet light. It will raise the blood calcium to normal or even above normal when given by mouth, and for this reason, has an advantage over parathyroid extract, which is given hypodermically. The effect of parathyroid extract is of short duration, not over forty-eight hours. When dihydrotachysterol is used, the blood calcium does not begin to rise until after forty-eight hours, reaches a maximum in about one week, and decreases in one to three weeks after its use is discontinued. When too much dihydrotachysterol is used, the patient complains of asthenia, nausea, vomiting, headache, stupor, thirst, or skin rash. The method of management of a patient under this treatment, with frequent check on the blood chemistry or urine, is described by Albright.<sup>2</sup> The urine test can easily be performed by the patient at home. Sulkowitch reagent is added to urine, and the observation of a fine, white precipitate with a normal excretion of calcium is indicative of a normal blood calcium. If the solution remains clear, hypocalcemia is present. In hypercalcemia the solution becomes milky with increased output of calcium.

Hypoparathyroidism causes low blood calcium, high blood phosphorus, decreased excretion of calcium in the urine, and positive calcium balance (less excretion than intake). What becomes of the calcium that is ingested is a mystery, since density of the bones is not evident on radiologic examination. It has been suggested that the calcium is deposited in the tissues. As the blood calcium goes down, the phosphorus goes up, and vice versa, so that it is necessary to decrease the blood phosphorus by a diet low in phosphorus in order to facilitate raising the calcium. While dihydrotachysterol raises the blood calcium, it does not mobilize the calcium in the blood by removing it from the bones, as is the case when parathyroid extract is used. Dihydrotachysterol will not prevent or cure rickets as irradiated ergosterol does, so that the latter is necessary to improve bone quality. It is better, therefore, to combine vitamin D, calcium, and dihydrotachysterol in the treatment of parathyropivic tetany.

The dosage of dihydrotachysterol is not well established. It varies with the amount of the gland removed and the degree of regeneration. If all parathyroid tissue has been removed, the dosage is more constant. Adams<sup>1</sup> reported a case of short duration following subtotal thyroidectomy in which the patient was symptom free on 1 c.c. twice a week. Newman<sup>5</sup> reported a case of parathyropivic disease with tetany of seven years' duration in which 1 c.c. of dihydrotachysterol daily was necessary in spite of the fact that the blood calcium ranged from 8 mg. to 12.6 mg. for each 100 c.c. of blood before treatment. The use of the drug in idiopathic tetany<sup>3, 4</sup> and scleroderma<sup>6</sup> is somewhat different from that following accidental removal of the parathyroids.

The case presented here is unusual and interesting for the following reasons: (1) absence of parathyroid tissue and the appearance of tetany any time treatment is discontinued; (2) dosage of dihydrotachysterol; (3) persistence of considerable laryngeal stridor even with a blood calcium of 9 mg.; (4) further improvement of laryngeal stridor with thyroid extract; (5) decalcification of jawbone; and (6) injury to the recurrent laryngeal nerve.



## REPORT OF CASE

The patient, a schoolteacher, aged 46 years, was referred for treatment on August 26, 1939. She complained of cramps in her arms and hands. During the spasms she could not straighten out her hands and fingers. There was extreme weakness with the attacks which were relieved only by an injection of parathyroid extract about every other day. She talked mainly in a whisper, but occasionally made rather high-pitched noises. Nervousness and irritability were pronounced. Her ankles were slightly swollen.

She gave a history of thyroidectomy in July, 1928. In June, 1939, a large adenoma was removed from the region of the right upper part of the thyroid, in which was imbedded the right upper parathyroid. The tetany first appeared following the latter operation. Other operations included tonsillectomy, appendectomy, uterine suspension, and a few dental extractions.

She stated that the goiter had originally appeared during puberty. Following thyroidectomy she felt well until 1932, when she had a nervous breakdown. The history from 1932 to 1939 was supplied by several doctors who had treated her during that period. The following basal metabolism readings were recorded during these years: 1932, +31, +24, +10, +15, +28, +30; 1933, +26, +26; 1934, +25; 1935, +14, +14, +35; 1936, +31, +39; 1937, +19, +19, +24, +28; 1938, +30; and 1939, +35, +44. The Wassermann test, urinalysis, and blood count were normal in 1934. A small mass became evident in the region of the upper half of the right lobe of the thyroid, and continued to increase in size. The patient presented symptoms of exhaustion, nervousness, pounding of the heart, protrusion of the eyeballs, loss of weight, and slight tremor of the fingers. Her pulse rate varied from 100 to 140; her blood pressure was 130 systolic and 75 diastolic. The menstruation was regular every twenty-eight days. The diagnosis was made of chronic nervous exhaustion, with adenoma of the right lobe of the thyroid and mild hyperthyroidism. Removal of the adenoma was advised by several doctors, but the patient preferred medical treatment and was given Lugol's solution, sedatives, and other treatment at various times until she submitted to removal of the adenoma.

Physical examination on the first visit here was essentially normal. The scar on the neck was slightly tender. The pulse rate was 84. Medication for each day consisted of one vitamin ABDG capsule, 6 tablets of calcium gluconate of 15 grains each, 6 tablets of desiccated parathyroid of 0.1 grain each, and 60,000 U.S.P. units of irradiated ergosterol. She was receiving parathyroid injections about every other day.

In September, 1939, dihydrotachysterol, 1 c.c. every three days, was added to the treatment and later increased to 1 c.c. daily, since the blood calcium was still 7.5 mg. for each 100 c.c. of blood. The patient was started on a high-calcium, low-phosphorus diet. The parathyroid injections were decreased to two a week and later to one a week. She began feeling stronger and gaining weight and was advised to start walking for exercise. Her basal metabolism was +12, her urinalysis was negative, and her blood pressure was 88 systolic and 60 diastolic.

In October she was much better, and showed some improvement in the voice and no swelling of the ankles. Since she was markedly nervous it was still necessary for her to rest all the time. With exercise the muscles between the shoulders would cramp, but there was no definite tetany; she had discontinued the parathyroid injections and tablets, as well as the irradiated ergosterol. At night during sleep she made sounds resembling snoring that kept her husband awake. One-half grain of phenobarbital at bedtime was prescribed. Her blood pressure was 98 systolic and 68 diastolic, her blood calcium was 9 mg., and her basal metabolism rate averaged -9.

In November and December she showed general improvement, but as her voice improved the breathing noises at night increased. She tried reducing the dihydrotachysterol from 15 minims to 10 minims daily, but her condition became worse and she had to resume taking 1 c.c. daily. Daily urine tests with the Sulkowitch reagent were started, and there was a fine, white precipitate most of the time, although occasionally the urine was clear or milky. Her blood calcium was 8 mg., her basal metabolic rates were -10 and -25; red blood cells 4,270,000, hemoglobin 74 per cent, and the urinalysis negative. Her weight increased to 147 pounds. The hair on her legs, head, and eyebrows, which had turned white, began to get darker. One-fourth grain of thyroid was added to the treatment.

In January and February, 1940, she showed general sluggishness, with some cramps in the legs and some shortness of breath. Her blood pressure was 100 systolic and 68 diastolic, her blood calcium was 8.5 mg., her pulse rate was 70 to 86, she weighed 151½ pounds. The urinalysis was negative. The thyroid extract was increased to 0.5 grain daily. She discontinued taking vitamin D. By March her voice had improved considerably. It was felt that the recurrent laryngeal nerve had regenerated, but there was now a loud crowing noise on inspiration and shortness of breath on climbing stairs. She stopped taking the thyroid extract, since she thought that she felt better without it. An electrocardiogram showed marked myocardial damage. Urinalysis was normal. She was advised to continue resting and to eat more fruits and vegetables and less of fattening foods. A postcard arrived in April, 1940, stating that she had stopped the dihydrotachysterol for ten days, but had a bad attack of tetany and had to take an injection of parathyroid. She had severe cramps, and could not straighten out her hands. The dihydrotachysterol was resumed.

The patient was not heard from again until July. The drops had been discontinued for three weeks. The tetany with carpopedal spasm had reappeared, and she had difficulty in breathing with loud tracheal stridor. A few injections of parathyroid helped to relieve the cramps, but there was considerable weakness and nervousness. She was placed in the hospital where she remained for two weeks. She made an uneventful recovery on 1 c.c. of dihydrotachysterol daily. Examinations by a surgeon, otolaryngologist, and neurologist were all essentially normal. The vocal cords were functioning normally, and all agreed with the diagnosis of tetany and laryngeal stridor due to parathyroprivia. The eyes were tested and glasses were fitted to correct a slight visual disturbance.

On the day the dihydrotachysterol was resumed, the blood calcium was 5.3 mg.; a few days later it was 7.2 mg., with 6.5 mg. of phosphorus. The hemoglobin was 69 per cent, red blood cells 4,380,000, color index 0.8, white blood cells 9,000, platelets and differential cells normal. The temperature ranged between 97.4° and 99° F., usually slightly subnormal. In August the calcium tablets were discontinued, and two teaspoonfuls of powdered calcium gluconate, three times daily, were given in their place. Marked laryngospasm and tracheal stridor continued in spite of this, although both symptoms improved to some extent. The urino tested with Sulkowitch reagent was always milky. Dental x-ray examination showed some decalcification of the jawbones. A roentgenogram of the spine showed no general decalcification. The blood pressure was 110 systolic and 72 diastolic. The basal metabolic rate was -27.

In September the patient was much better; she looked rested and felt stronger, but she still had dyspnea with loud inspiration noises. When she was asleep, her breathing could be heard all over the house. Menstruation was still regular every month. Foods containing phosphorus were eliminated from the diet. Irradiated ergosterol, 3,500 U.S.P. units, was given after each meal. Blood calcium was 8.5 mg. At the beginning of the second week in September, 1 grain of desiccated thyroid was given daily. Blood calcium was 7 mg., and blood phosphorus was 4.1 mg. The pulse rate was 80, and the blood pressure was 100 systolic and 72 diastolic.

On Monday, September 16, 1940, the patient was feeling much better and started to work. By September 20 she was feeling stronger, although she had finished one week of work. She was breathing easier and with much less noise. Her pulse rate was 80. On September 27 she was feeling very well; she had no dyspnea and no stridor at all when sitting, and only slight musical noise on inspiration during exercise. At the end of the second week of work she felt she had succeeded in her effort to get back on the job. This was the first time she had worked since June, 1939. During October she still continued in relatively good health. Her blood calcium was 8.3 mg. and her blood phosphorus was 5.3 mg.

#### DISCUSSION

In the case just presented, the laryngeal stridor was the most difficult symptom to overcome. It was necessary to give intensive treatment with a combination of drugs and diet to improve the calcium metabolism before improvement occurred. The dihydrotachysterol relieved the irritability and spasm

of the muscles, and the thyroid improved the muscle tone and use. These two drugs were the most useful in the treatment of the case. It was necessary to keep a close check on the diet. The dosage of all medication essential to create and to maintain a state of good health was larger than expected. The treatment was expensive, since the monthly allowance of 30 c.c. of dihydrotachysterol plus the other drugs averaged about \$20.00.

A milky urine when tested with Sulkowitch reagent did not mean that hypercalcemia was present. When larger doses of calcium were given by mouth to this patient, the urine test was always milky, although the blood calcium was only 8 or 9 mg. for each 100 c.c. of blood. The patient is going to try to take less of the calcium gluconate with the same amount of the other drugs since she is probably taking more calcium than is necessary for maximum improvement. Of course, there is no danger of a high blood calcium if the urine is clear or slightly cloudy. The test is of diagnostic value before treatment, and it can be used during treatment if smaller amounts of calcium are given. When the urine test is milky, a blood test is necessary to determine the exact level of the blood calcium.

#### SUMMARY

The treatment of a case of parathyroid tetany with injury to the recurrent laryngeal nerve is presented. The recurrent laryngeal nerve regenerated in a few months, but the deficiency in parathyroid hormone continued after one and one-half years. Maximum improvement was brought about only after intensive treatment with a low phosphorus diet, calcium gluconate, irradiated ergosterol, dihydrotachysterol, and thyroid extract. Dihydrotachysterol raised the blood calcium, prevented the tetany, and partially decreased the laryngeal stridor. The thyroid extract raised the basal metabolism and further improved the laryngeal stridor. The minimum dosage of dihydrotachysterol was 1 c.c. daily. There was decalcification of the jawbones, but no general decalcification of the skeleton.

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## THE COMPARATIVE PHYSIOLOGIC VALUE OF INJECTED CAROTENE AND VITAMIN A\*

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MANY workers have observed that carotene or vitamin A may be biologically active when injected into experimental animals (Wollman and Vogliano;<sup>1</sup> Koehne and Mendel;<sup>2</sup> Wolff, Overhoff, and van Eekelen;<sup>3</sup> von Euler and Rydholm;<sup>4</sup> Rydholm;<sup>5</sup> Gordon and Titherington;<sup>6</sup> Chu and Coady;<sup>7</sup> Laseh;<sup>8</sup> Greaves and Schmidt;<sup>9, 10</sup> Guilbert and Hart;<sup>11</sup> Wilson, Ahmad, and Majumdar;<sup>12</sup> Phillips and Bohstedt<sup>13</sup>). Parenteral carotene and vitamin A have also been reported to be effective in human therapeutics (Blegvad;<sup>14</sup> Edmund;<sup>15</sup> Bloch;<sup>16, 17</sup> Buschke;<sup>18</sup> Josephson and Freiburger<sup>19</sup>). However, there is also abundant evidence that injected vitamin A is not always efficiently utilized. Funk<sup>20</sup> stated that vitamin A given parenterally was without any activity. Wollman and Vogliano<sup>1</sup> observed that repeated injections of cod-liver oil or butter oil were not tolerated well, since no growth resulted until after injections were stopped.

Koehne and Mendel<sup>2</sup> observed only temporary remission of deficiency symptoms with the accumulation of much foreign fat at the site of injection when butter oil or cod-liver oil were injected into depleted rats. Von Euler and Rydholm<sup>4</sup> found that 0.050 mg. of carotene injected daily was inadequate for growth in depleted rats, although much smaller amounts given orally were known to restore growth. Chu and Coady<sup>7</sup> also reported better results with carotene given orally than with that given parenterally. Drummond and co-workers<sup>21-24</sup> failed to find any storage of vitamin A in the livers of rats and cats when carotene, emulsified in glucose solution, was injected intravenously. In one experiment with rabbits, however, some vitamin A was stored. This relative inertness of parenteral carotene has also been observed by Ahmad, Grewal, and Malik<sup>25</sup> in their work with rats and dogs.

Furthermore, preliminary experiments of our own indicated that injected carotene was not converted into vitamin A efficiently. Rats with stores of 175 blue units per liver were fed a fat-free diet, and then given intraperitoneal injections of large amounts of an aqueous suspension of colloidal carotene. No vitamin A could be detected in the livers after five weeks, although much carotene was found at the site of injection, adherent to the liver.

Parenteral administration of vitamin A sometimes appears to be indicated in human therapy (Blegvad;<sup>14</sup> Bloch;<sup>16, 17</sup> Gordon and Titherington;<sup>6</sup> Edmund

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and Clemesen;<sup>20</sup> Finkelstone, Howard, and Paris;<sup>27</sup> Decourt;<sup>28</sup> Buschke;<sup>18</sup> Josephson and Freiburger<sup>19</sup>). However, experimental data on the relative efficiencies of the various means of administration are lacking even with animals. We have, therefore, carried out experiments with rats, using several biological criteria, such as cure of ophthalmia, duration of cure, and growth and survival time, together with data on the carotene and vitamin A content of the tissues.

#### METHODS

Rats 3 weeks old and 35 to 50 gm. in weight were fed a basal ration composed of casein (heated at 95° C. for fourteen days) 18 per cent, Osborne and Mendel salt mixture (as modified by Hawk and Oser<sup>29</sup>) 4 per cent, brewer's yeast 7.9 per cent, irradiated brewer's yeast 0.1 per cent, hydrogenated fat\* 10 per cent, and sucrose 60 per cent. Food and water were given ad libitum throughout both the preliminary and experimental periods. Supplements of carotene or vitamin A were administered when the animals showed a loss in weight and the initial stages of ophthalmia. The supplements were given in the form of colloidal water suspensions or as oil solutions. Both were fed as well as injected. The amounts given ranged from 75 to 1,500  $\mu$ g. The volumes of the doses did not exceed 0.25 c.c. for the oil solutions, and 1 c.c. for the colloidal suspensions. A detailed account of the various doses administered is given in the tables of results. In the evaluation of the biological response primary emphasis was placed upon the cure of ophthalmia because of the possibility that repeated injections might interfere with growth.<sup>1</sup> At the end of the experimental period the animals were killed and the tissues were analyzed for carotene and vitamin A.

The aqueous colloidal suspensions of carotene were prepared by slowly adding carotene in acetone solution to hot (90° C.) water agitated with a stream of nitrogen. The preparations were made fresh every three days and filtered to remove visible particles. A similar procedure was used for the preparation of aqueous colloidal solutions of vitamin A. The source of vitamin A was halibut-liver oil.† Its unsaponifiable matter was dissolved in methyl alcohol, chilled to -10° C., and filtered. The filtrate was dried under nitrogen and reduced pressure, then dissolved in acetone, and slowly added to hot (90° C.) water agitated with nitrogen.

For its quantitative determination the carotene was taken up in chloroform, and the intensity of absorption was measured at 485 and 460  $m\mu$  in a Bausch & Lomb spectrophotometer. Animal tissues were first dissolved in 8 per cent potassium hydroxide at room temperature overnight, and then 2 volumes of water with sufficient alcohol to make a concentration of 15 per cent alcohol were added. The carotene was extracted with peroxide-free ether, the extracts were washed with water and evaporated in vacuo, and the residue was dissolved in chloroform.

A similar procedure was employed in the preparation of extracts suitable for the determination of vitamin A by the colorimetric method of Carr and Price.<sup>30</sup>

\*A mixture of partially hydrogenated vegetable fats marketed under the trade name "Spry."

†The halibut-liver oil was a commercial brand known as Haliver Oil, furnished by the Abbott Laboratories.

## PARENTERAL ADMINISTRATION OF CAROTENE

(a) *Physiologic Requirements*.—When aqueous colloidal carotene was injected in amounts equivalent to 375, 750, or 1,500  $\mu\text{g}$  per rat per week, ophthalmia was cured and growth was restored (Table I). Growth was equally good with injections of 750 or 1,500  $\mu\text{g}$  a week, with an approximate gain of 60 Gm. in weight in five weeks. This was also the rate of growth observed when 75  $\mu\text{g}$  of colloidal carotene were given weekly by stomach tube or when 7  $\mu\text{g}$  of carotene were fed in oil solution by dropper. Thus, the amount of carotene necessary to produce a given biological response may vary by over a hundredfold, depending upon the mode of administration. Lesser amounts of carotene produced lesser gains in weight, e.g., the intraperitoneal injection of 375  $\mu\text{g}$  of colloidal carotene per week resulted in a gain of only 37 Gm. in five weeks, whereas 75  $\mu\text{g}$  per week failed to restore growth, to cure ophthalmia, or to maintain life. At our levels of administration (Table I) no difference in the response of animals injected subcutaneously or intraperitoneally was noted. Furthermore, carotene injected in oil appeared to be as effective as colloidal carotene. The presence of 5 per cent of glucose in the suspension of colloidal carotene failed to affect the results in any way.

TABLE I  
EFFECT OF CAROTENE ADMINISTERED IN VARIOUS WAYS

| LEVEL OF CAROTENE GIVEN ( $\mu\text{g}/\text{WK.}$ ) | MEDIUM             | METHOD OF ADMINISTRATION           | AVERAGE GAIN IN WEIGHT AT 5 WK. (Gm.) | AVERAGE CAROTENE IN LIVER AT DEATH ( $\mu\text{g}$ ) | AVERAGE CAROTENE AT SITE OF INJECTION ( $\mu\text{g}$ ) |
|--|--------------------|------------------------------------|---------------------------------------|--|---|
| 1,500  | Water              | (a) Parenteral*<br>Intraperitoneal | 62                                    | 365 (211 to 507)                                     | 1,807 (1,605 to 1,990)                                  |
| 1,500  | Water              | Subcutaneous                       | 63                                    | 106 ( 14 to 153)                                     | 1,860 (1,560 to 2,220)†                                 |
| 750  | Water              | Intraperitoneal                    | 57                                    | 185 (112 to 246)                                     | 750 ( 585 to 1,030)                                     |
| 750  | Water              | Subcutaneous                       | 62                                    | 129 (118 to 141)                                     | 543 ( 480 to 600)                                       |
| 750  | Water<br>(glucose) | Intraperitoneal                    | 69                                    | 184 (126 to 274)                                     | 619 ( 576 to 658)                                       |
| 750  | Oil                | Intraperitoneal                    | 67                                    | 45 ( 21 to 87)                                       | 1,766 (1,185 to 2,140)                                  |
| 375  | Water              | Intraperitoneal                    | 37                                    | 44 ( 6 to 90)  | 40 ( 15 to 75)  |
| 75   | Water              | Intraperitoneal                    | All dead                              | 5 (trace to 15)                                      | 4 ( 2 to 8)   |
| 75   | Oil                | Intraperitoneal                    | All dead                              | 8 (trace to 11)                                      | 153 ( 115 to 229)                                       |
| 750  | Water              | (b) Oral                           | 91                                    | —  | —   |
| 75   | Water              | Stomach tube                       | 58                                    | —  | —   |
| 7  | Oil                | Dropper                            | 60                                    | —  | —   |

\*At least 3 animals were used at each level.

†Some carotene was also found in the mesentery and omentum.

The potency of colloidal carotene given orally was demonstrated not only by the amount of growth elicited, but also by the fact that 750  $\mu\text{g}$  given each week for five weeks resulted in the hepatic storage of 567 blue units of vitamin A. By way of contrast the injection of 1,500  $\mu\text{g}$  of colloidal carotene failed to yield any stores of vitamin A. Carotene injected in oil likewise failed to produce stores of vitamin A.

Injected carotene, however, accumulated in large amounts in tissues other than the liver. Both the amount and the location of these deposits depended upon the method of administration. After intraperitoneal injection, carotene was found in the omentum, mesentery, lymph nodes, and in the abdominal fat and

membranes surrounding the liver. After subcutaneous injection, carotene was found in similar areas and also at the site of injection (Table I). Oil solutions of carotene given intraperitoneally remained in the abdominal cavity for a considerable period of time, and much less carotene migrated to the liver than after the injection of the colloidal preparation (Table I). Apparently carotene was treated in part as a foreign substance because young rats injected with India ink showed a distribution of carbon particles similar to that of carotene. The presence of carotene at the site of injection has been reported previously (van den Bergh, Muller, and Broekmeyer;<sup>31</sup> Rydholm;<sup>5</sup> Rea and Drummond;<sup>21</sup> Wilson, Ahmad, and Majumdar<sup>12</sup>).

TABLE II

AVAILABILITY OF CAROTENE DEPOSITS FORMED BY PARENTERAL ADMINISTRATION

| TOTAL CAROTENE GIVEN ( $\mu$ g)                  | MEDIA | METHOD OF ADMINISTRATION              | DAYS TO REAPPEAR-ANCE OF OPHTHALMIA | DAYS TO MAXIMUM INCREASE IN WEIGHT | DAYS OF SURVIVAL  |
|--|-------|---------------------------------------|-------------------------------------|------------------------------------|-------------------|
| 1,250 (in 5 days)                                | Water | <i>Parenteral*</i><br>Intraperitoneal | 30<br>(25 to 31)                    | 22<br>(16 to 34)                   | 39†<br>(28 to 63) |
| 1,250 (in 5 days)                                | Water | Subcutaneous                          | 25<br>(21 to 30)                    | 21<br>(13 to 31)                   | 37†<br>(28 to 54) |
| 1,250 (in 5 days)                                | Oil   | Intraperitoneal                       | 36‡<br>(34 to 38)                   | 38<br>(25 to 51)                   | 39<br>(20 to 58)  |
| 250  | Water | Intraperitoneal                       | 16§                                 | 11<br>(9 to 13)                    | 16<br>(5 to 26)   |
| 1,250 (in 5 days)                                | Water | <i>Oral</i><br>Stomach tube           | 71  <br>(61 to 81)                  | 50<br>(36 to 58)                   | 90<br>(54 to 110) |
| 50 (in 5 days)                                   | Oil   | Dropper                               | 26<br>(21 to 29)                    | 21<br>(11 to 29)                   | 30<br>(24 to 36)  |
| Had been<br>375 $\gamma$ of carotene<br>per week | Water | <i>Parenteral</i><br>Intraperitoneal  | 21<br>(15 to 23)                    | 7<br>(3 to 9)                      | 28<br>(20 to 35)  |
| 750 $\gamma$ of carotene<br>per week             | Water | Intraperitoneal                       | 20<br>(15 to 28)                    | 17<br>(8 to 34)                    | 33<br>(21 to 57)  |
| 75 $\gamma$ of carotene<br>per week              | Water | <i>Oral</i><br>Stomach tube           | 29<br>(24 to 34)                    | 13                                 | 45<br>(26 to 64)  |

\*At least 4 rats were used at each level.

†Some rats were killed when losing weight and showing severe ophthalmia, others died.

‡Two rats died without relapse of symptoms.

§Two rats showed no remission of ophthalmia; another died without relapse of symptoms.

||One rat died without relapse of symptoms.

(b) *Availability of Carotene Deposits.*—The presence of large deposits of injected carotene raised the question of their availability. Accordingly, rats were given large injections of carotene and were then continued on the basal ration with observations on growth, cure of ophthalmia, reappearance of ophthalmia, and time of death. Details of dosage, mode of administration, and results are given in Table II. The liver and various other tissues were analyzed for carotene.

Animals that were given 5 injections totaling 1,250  $\mu$ g of carotene grew for a short time, and their ophthalmia disappeared (Table II), but within a month ophthalmia reappeared with loss of weight until death supervened. In

all cases mutilized carotene was found in the livers and at the site of injection (Table III). Animals injected with only 250  $\mu\text{g}$  of carotene were variable in therapeutic response, but deposits of carotene were invariably found at death. No essential difference in result was obtained when carotene was injected in oil solution or in colloidal suspension.

TABLE III

CAROTENE FOUND IN RATS AFTER INJECTION OF 1,250 GAMMA OF CAROTENE OVER A FIVE-DAY PERIOD\*

| METHOD OF ADMINISTRATION | MEDIA | TIME AFTER LAST INJECTION (DAYS) | TOTAL CAROTENE IN LIVER ( $\mu\text{g}$ ) | CAROTENE AT SITE OF INJECTION ( $\mu\text{g}$ ) |
|--------------------------|-------|----------------------------------|---|---|
| Intraperitoneal          | Water | 2                                | 35  | 147   |
| Intraperitoneal          | Water | 30 $\pm$ 1                       | (10 to 72)<br>22<br>(4 to 92)             | (104 to 183)<br>65<br>(8 to 121)                |
| Subcutaneous             | Water | 2                                | 11  | 138   |
| Subcutaneous             | Water | 30 $\pm$ 1                       | (8 to 15)<br>4<br>(traces to 18)          | (127 to 151)<br>30<br>(4 to 182)                |
| Intraperitoneal          | Oil   | 30 $\pm$ 1                       | 16<br>(traces to 42)                      | 267<br>(138 to 352)                             |

\*Each value is an average of 3 or more rats.  
†Animals had died of vitamin A deficiency.

To determine whether large deposits of injected carotene were more effective biologically than small deposits, we used animals which had been injected with 375 or 750  $\mu\text{g}$  of carotene weekly for five weeks. Carotene deposits averaged 935  $\mu\text{g}$  in the latter (Table I) as compared with 182  $\mu\text{g}$  in animals injected only over the five-day period (Table III). Nevertheless, the behavior of the two groups of animals was essentially the same when they were continued on the basal low vitamin A diet. The restoration of growth and the cure of ophthalmia were temporary, and death occurred in an average of thirty-three days in both groups. Apparently the accumulated carotene was practically unavailable. No carotene was found in the urine or feces after its injection.

#### PARENTERAL ADMINISTRATION OF VITAMIN A

In view of the inefficient utilization of injected carotene, parallel experiments were performed with vitamin A.

(a) *Physiologic Requirements*.—Vitamin A was injected as an aqueous colloidal suspension or as a solution of halibut-liver oil in cottonseed oil. Seven hundred and fifty blue units injected weekly or fed by stomach tube restored growth, cured ophthalmia, and produced hepatic stores (Table IV). The results were essentially the same whether the colloidal vitamin was injected subcutaneously or intraperitoneally, or fed by stomach tube. When the vitamin was injected in oil solution, however, somewhat better growth resulted than when the colloidal vitamin was injected.

A general consideration of all the results obtained indicated that the efficiency of injected vitamin A was approximately equal to that of vitamin A given orally, as judged by the criteria of growth, cure of ophthalmia, and hepatic storage. At lower levels of administration, 30 and 75 blue units per week, the



biological response depended to some extent on the vehicle in which the vitamin was administered and also on the method of administration. Aqueous colloidal vitamin A was more effective when injected than when given orally (Table IV); oil solutions, on the contrary, were more effective when given by mouth.

TABLE IV  
EFFECT OF VITAMIN A ADMINISTERED IN VARIOUS WAYS

| LEVEL OF VITAMIN A GIVEN (B.U./WK.)* | MEDIA | METHOD OF ADMINISTRATION           | AVERAGE GAIN IN WEIGHT AT 5 WEEKS (GM.) | AVERAGE HEPATIC STORES (B.U.) |
|--------------------------------------|-------|------------------------------------|---|-------------------------------|
| 750                                  | Water | (a) Parenteral†<br>Intraperitoneal | 59                                      | 386                           |
| 750                                  | Water | Subcutaneous                       | 70                                      | 358                           |
| 750                                  | Oil   | Intraperitoneal                    | 115                                     | 196‡                          |
| 75                                   | Water | Intraperitoneal                    | 52                                      | 0                             |
| 30                                   | Water | Intraperitoneal                    | 21 (1 dead)                             | 0                             |
| 30                                   | Oil   | Intraperitoneal                    | 14 (2 dead)                             | 0                             |
| 750                                  | Water | (b) Oral<br>Stomach tube           | 67§                                     | 412                           |
| 75                                   | Water | Stomach tube                       | -12 (2 dead)                            | 0                             |
| 30                                   | Water | Stomach tube                       | -10 (2 dead)                            | 0                             |
| 30                                   | Oil   | Dropper                            | 63                                      | 0                             |

B.U. = Blue unit.

\*The vitamin A was administered three times weekly for a five-week period.

†Each value is an average of 4 or more rats.

‡Some vitamin A and oil from the abdominal cavity were unavoidably included.

§One rat responded poorly throughout the experimental period.

The foregoing results may be rationalized on two assumptions: first, that colloidal vitamin A is relatively unstable to the acids of the digestive tract; and secondly, that the vitamin is more readily mobilized from the injected aqueous suspension than from an injected oil solution. The colloidal vitamin A was presumably present as the alcohol as a result of previously saponifying the halibut-liver oil, which contains the vitamin in the form of esters (Hickman<sup>32, 33</sup>). This fact may be noteworthy because vitamin A alcohol and ester are reported to have unequal biological activities when given by mouth (Moll and Reid<sup>34</sup>). It is of interest that Gordon and Titherington<sup>6</sup> observed that vitamin A was somewhat more effective on parenteral administration than when given orally, whereas Greaves and Schmidt<sup>10</sup> reached the opposite conclusion.

In contrast with carotene, most of the injected vitamin A accumulated in the liver rather than at the site of injection. For example, when 750 blue units of colloidal vitamin A were injected intraperitoneally and weekly for five weeks, 105 to 140 units remained in the peritoneal cavity, and 386 units appeared in the liver. On subcutaneous injection 20 blue units remained at the site of injection, and 358 units appeared in the liver. Injected oil solutions, however, persisted at the site of injection or in the abdominal cavity. Lipoidal masses containing 1,125 to 1,797 blue units were found adhering to the liver, although the liver tissue contained relatively little (Table IV).

(b) *Availability of Vitamin A Deposits.*—The question remained whether stores of vitamin A produced by injection could be utilized readily, or whether, like injected carotene, they were inert. Deficient rats, therefore, were given large injections of vitamin A and then continued on the basal diet. Notations were made on the reappearance of ophthalmia and the time of survival (Table V).

Two hundred and fifty blue units of colloidal vitamin A administered in one dose were insufficient to produce hepatic stores, although ophthalmia was cured and growth was promoted for a short time; following this, ophthalmia reappeared, growth failed, and death resulted. The response was essentially the same whether the colloid was given subcutaneously, intraperitoneally, or orally. However, a better response was obtained with injections in oil (Table V). A total of 1,250 blue units of colloidal vitamin produced the following hepatic stores over a five-day period: 142 blue units after intraperitoneal injection, 121 units after subcutaneous injection, and 149 units when fed by stomach tube.

TABLE V  
AVAILABILITY OF VITAMIN A STORES OF RATS\*

| TOTAL<br>VITAMIN A<br>GIVEN (B.U.) | MEDIA | METHOD OF<br>ADMINISTRATION | AVERAGE DAYS TO<br>REAPPEARANCE OF<br>OPHTHALMIA | SURVIVAL<br>PERIOD<br>(DAYS) |
|------------------------------------|-------|-----------------------------|--|------------------------------|
| 1,250 (in 5 days)                  | Water | (a) Parenteral              |  |                              |
| 1,250 (in 5 days)                  | Water | Intraperitoneal             | 79† (76 to 81)                                   | 58 (63 to 108)               |
| 250 (1 injection)                  | Water | Subcutaneous                | 64 (44 to 93)                                    | 87 (71 to 113)               |
| 250 (1 injection)                  | Water | Intraperitoneal             | 27 (13 to 46)                                    | 38 (13 to 75)                |
| 250 (1 injection)                  | Water | Subcutaneous                | 37‡ (32 to 40)                                   | 39 (15 to 69)                |
| 250 (1 injection)                  | Oil   | Intraperitoneal             | 49† (31 to 63)                                   | 62 (39 to 88)                |
| 1,250 (5 doses)                    | Water | (b) Oral                    |  |                              |
| 250 (1 dose)                       | Water | Stomach tube                | 53† (47 to 60)                                   | 71 (58 to 79)                |
|                                    |       | Stomach tube                | 24§ (13 to 40)                                   | 36 (18 to 62)                |

\*Each value is an average of 1 or more rats.

†One animal died without relapse of symptoms.

‡Two animals died without relapse of symptoms.

§One animal showed no remission of ophthalmia.

Despite the similarity in the stores produced, the vitamin appeared to be more effective physiologically when injected than when given orally; injected animals lived for eighty-eight days as compared with seventy-one days for those given the vitamin orally (Table V). Ophthalmia was deferred for seventy-two days in the injected animals as compared with fifty-three for the latter. At death no stores of vitamin A were found either in the liver or at the site of injection. Vitamin A administered parenterally, therefore, was apparently utilized at least as efficiently as when given orally.

#### SUMMARY

Rats deficient in vitamin A were injected intraperitoneally or subcutaneously with carotene in aqueous colloidal suspensions or in oil solution. Growth was restored and symptoms of ophthalmia were cured, but the amount needed was ten to one hundred times as great, and the effect was less prolonged than when carotene was given orally. Parenteral carotene failed to yield stores of hepatic vitamin A even when 1.5 mg. were injected weekly for five weeks. Particles of injected carotene were found at the sites of injection, and also in the liver, omentum, and lymph nodes in the peritoneal cavity. Carotene in these areas was only partially available to the animal, since deficiency symptoms and death occurred while considerable amounts of carotene were still in evidence.

Much of the injected carotene could not be traced; it was not converted into vitamin A nor was it found in the excreta.

Vitamin A, in contrast to carotene, was utilized about as effectively when injected as when given orally. Aqueous colloidal preparations were utilized best when injected; oil solutions were utilized best when given orally.

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## THE INFLUENCE OF A PAROTID EXTRACT ON THE BLOOD SUGAR AND STRUCTURE OF THE PANCREAS OF THE RAT\*

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THE role of the parotid gland in carbohydrate metabolism has been the subject of controversy. Blood sugar-raising substances have been obtained from the parotid gland by Hiki and co-workers.<sup>1</sup> Takaes,<sup>2</sup> however, reported hypoglycemias following the injection of ether and alcohol extracts of this gland. Birnkrant<sup>3</sup> demonstrated marked hypoglycemias in rats following total bilateral parotidectomies. Cahane and Cahane<sup>4</sup> described hypertrophy and hyperplasia of the islands of Langerhans following bilateral parotidectomies on two dogs. Aunap,<sup>5</sup> Dobrzaniecki, and Michalowski,<sup>6</sup> reported diminution of hyperglycemias following parotidectomies in depancreatized dogs. Zibordi<sup>7</sup> and Scollo<sup>8</sup> have also demonstrated a relationship between the pancreas and the parotid gland in their experimental animal work.

There also has been some clinical evidence to support a parotid-pancreas relationship. Of Flaum's<sup>9</sup> 27 patients with bilateral hypertrophy of the parotid gland, 16 had frank diabetes and 11 had incipient diabetes. He considers every patient presenting noninflammatory enlargements of the parotid gland a diabetic unless proved otherwise. Kenawy<sup>10</sup> found approximately 2 per cent of the poorer agriculture classes in Egypt suffering from a chronic, painless, noninfectious, noninflammatory enlargement of the parotid gland. Ten per cent of the cases he studied were diabetics. All presented bilateral swellings.

Both Ferretti<sup>11</sup> and Dobreff<sup>12</sup> stated that hypertrophy of the parotid glands is a result of a compensatory mechanism, i.e., the parotid glands take on the function of the pancreas in some instances of hypoinsulinism. On the other hand, the Cahanes, and Dobrzaniecki and Michalowski interpreted their findings on the basis of an anti-insulin parotid hormone.

Although the work of these authors indicates a carbohydrate-regulating mechanism related to the parotid gland, apparently conflicting reports and opposing interpretations have made difficult a proper evaluation of this mechanism.

The present study was, therefore, undertaken to determine the effects of parotid extracts on tissues and on blood sugar levels. Blood-sugar raising sub-

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stances have been prepared from the urine of diabetic patients by Werch and Altshuler,<sup>13</sup> and by Harrow and associates.<sup>14</sup> In order to determine whether a relationship exists between these blood-sugar raising substances isolated from the urine of diabetic patients and the parotid extracts used by the earlier workers, a parotid extract was prepared by modifying the method of Harrow and others in order to make it suitable for tissue work.

#### EXPERIMENTAL TECHNIQUES

(A) *Preparation of Extract.*—To 1,250 Gm. of fresh ground beeves' parotid gland from which the fat and fascia had been removed, 2,250 c.c. of distilled water were added. The mixture was digested for twelve hours at 10° C. in three-eighths its volume of 0.2 N sodium hydroxide, then filtered and centrifuged. The supernatant fluid was acidified to pH 5 with glacial acetic acid. Sixty cubic centimeters of 95 per cent alcohol, containing 30 Gm. of benzoic acid, were added for each liter of solution. The precipitate was sucked dry on a Buchner funnel, washed with alcohol and ether, dried in vacuo and weighed (precipitate I, weight 132 Gm.).

The crude extract was triturated with 12 c.c. of distilled water per gram and dialyzed at 4° to 10° C. for three days. The contents of the dialyzing membrane were evaporated in vacuo to a small volume and centrifuged. The dried sediment (precipitate II) weighed 104 Gm.

To the supernatant fluid from this centrifuged mixture, absolute alcohol was added to make a final 95 per cent solution. This solution was kept at 4° to 10° C. for thirty-six hours and then centrifuged. The sediment was washed with alcohol and ether and dried (precipitate III, weight 320 mg.). Precipitate III was composed of thin, highly refractile, monoclinic, tetragonal crystals, soluble in water and dilute alcohol, insoluble in absolute alcohol and ether, and partially soluble in acetone. It turned brown and gummy on standing.

(B) *Blood Sugar Determinations.*—Precipitates I, II, and III were injected intraperitoneally into male Long-Evans hooded rats, eight to twelve weeks old and weighing from 207 to 305 Gm. The rats were kept on a well-balanced constant diet. Blood samples were secured from the palmar veins. The modified microcolorimetric method of Benedict<sup>15</sup> was used for the sugar determinations. This method determines nonfermentable substances equivalent in reducing power to only 4 to 8 Gm. of glucose per 100 c.c. of blood.

For the glucose tolerance tests 1 Gm. of glucose per kilogram of rat weight was fed orally in a 50 per cent solution to the fasting rat. The rats were previously conditioned to medicine dropper feedings.

Six or more blood sugars were determined on each rat prior to the experimental procedures in order to establish normal means.

#### EXPERIMENTAL PROCEDURES AND RESULTS

(A) *Parotid Extracts.*—1. Precipitate I. Seventy-five milligrams of precipitate I were injected intraperitoneally into four fasting rats. Seven days later two of these rats were again injected with the same amount of extract, and fourteen days later one was reinjected. Fasting blood sugar levels were determined, and blood samples were secured in one hour, four hours, and twenty-four hours after injection.

In one hour the blood sugars showed an average increase of 87 per cent over the fasting blood sugars, ranging from 163 mg. per cent to 216 mg. per cent (Table I), representing a markedly significant rise (the sigma of the difference equals 12). Four hours after injection the blood sugars were still significantly higher than those of the fasting levels. In twenty-four hours all the blood sugars had returned to the preinjection range.

TABLE I  
EFFECTS OF PRECIPITATE I ON BLOOD SUGARS

| RAT NO.                    | MEANS OF SIX BLOOD SUGARS PRIOR TO INJECTION | FASTING BLOOD SUGAR PRIOR TO INJECTION | PRECIPITATE I INJECTED (MG.) | BLOOD SUGARS AFTER INJECTION (MG. %) |        |        |
|----------------------------|--|--|------------------------------|--------------------------------------|--------|--------|
|                            |  |  |                              | 1 HR.                                | 4 HR.  | 24 HR. |
| 14                         | 82± 4  | 90                                     | 75                           | 171                                  | 146    | 121    |
| 36                         | 91± 6  | 85                                     | 75                           | 179                                  | 116    | 88     |
| 35                         | 91± 3  | 103                                    | 75                           | 163                                  | 157    | 109    |
| Test repeated 7 days later | 91± 3  | 104                                    | 75                           | 166                                  | 130    | 97     |
| 30                         | 106± 4                                       | 92                                     | 75                           | 216                                  | 149    | 95     |
| Test 7 days later          | 106± 4                                       | 108                                    | 75                           | 188                                  | 139    | 94     |
| 15 days later              | 106± 4                                       | 97                                     | 75                           |                                      |        |        |
| Mean                       | 95± 2  | 97± 3                                  |                              | 171                                  | 149    | 94     |
| Mean increase              |  |  |                              | 178± 7                               | 111± 6 | 100± 4 |
| Mean per cent increase     |  |  |                              | 83± 7                                | 46± 5  |        |
|                            |  |  |                              | 87                                   | 45     |        |

TABLE II  
EFFECTS OF PRECIPITATE II ON BLOOD SUGARS

| RAT NO.                | MEANS OF SIX BLOOD SUGARS PRIOR TO INJECTION | AMOUNT INJECTED (MG.) | BLOOD SUGARS AFTER INJECTION (MG. %) |        |        |
|------------------------|--|-----------------------|--------------------------------------|--------|--------|
|                        |  |                       | 1 HR.                                | 4 HR.  | 12 HR. |
| 3                      | 94± 5  | 50                    | 139                                  | 148    | 123    |
| 14                     | 82± 5  | 50                    | 155                                  | 130    | 122    |
| 15                     | 100± 4                                       | 50                    | 164                                  | 117    | 126    |
| 29                     | 93± 6  | 50                    | 130                                  | 90     | 117    |
| 30                     | 106± 4                                       | 50                    | 128                                  | 103    | 109    |
| 51                     | 93± 4  | 50                    | 146                                  | 115    | 128    |
| 55                     | 88± 5  | 50                    | 166                                  | 106    | 123    |
| 60                     | 97± 5  | 50                    | 146                                  | 119    | 110    |
| 62                     | 90± 5  | 50                    | 160                                  | 108    | 110    |
| Mean                   | 93± 1  |                       | 150± 7                               | 117± 6 | 118± 3 |
| Mean increase          |  |                       | 57± 7                                | 24± 6  | 25± 3  |
| Mean per cent increase |  |                       | 61                                   | 26     | 27     |

2. Precipitate II. Fifty milligrams of precipitate II were injected intraperitoneally into each of ten rats daily for eight days. Samples of blood were secured on the eighth day immediately prior to the injection. Thereafter, one-hour, four-hour, and twelve-hour fasting samples were drawn from each rat. In one hour the blood sugars showed a 61 per cent average increase. The four-hour sample was 26 per cent above the mean blood sugars prior to the series of injections. Twelve hours after injection the same average increase was recorded (Table II). These average increases in blood sugars were statistically significant.

Eighteen glucose tolerance tests were done on 11 rats after they had received from one to twelve consecutive daily injections of 100 mg. of precipitate II (Table III). Fasting blood samples were secured immediately before injection. Fourteen rats were injected with 100 mg. of precipitate II just prior to the glucose tolerance test. Four rats were not injected immediately prior to the test, but received their last injection twenty-four hours previously. Two tests were done after one injection, two after three daily injections, eight after eight daily injections, four after nine daily injections, and two after twelve daily injections.

TABLE III  
GLUCOSE TOLERANCE TESTS OF RATS INJECTED WITH PRECIPITATE II

| RAT NO. | NO. OF CONSECUTIVE DAILY INJECTIONS | MEAN OF SIX BLOOD SUGARS PRIOR TO INJECTIONS | FASTING BLOOD SUGARS | PRECIPITATE II PRIOR TO TEST (MG.) | BLOOD SUGARS FOLLOWING INJECTION (MG. %) |       |        |       |       |
|---------|-------------------------------------|--|----------------------|------------------------------------|--|-------|--------|-------|-------|
|         |                                     |  |                      |                                    | ½ HR.                                    | 1 HR. | 1½ HR. | 2 HR. | 3 HR. |
| 49      | 1                                   | 97± 4  | 97                   | 100                                | 160                                      | 216   | 196    | 202   | 128   |
| 2       | 1                                   | 93± 2  | 94                   | 100                                | 189                                      | 169   | 158    | 139   | 128   |
| 15      | 3                                   | 100± 4                                       | 106                  | 100                                | 153                                      | 178   | 162    | 146   | 108   |
| 14      | 3                                   | 82± 5  | 117                  | 100                                | 171                                      | 162   | 160    | 128   | 115   |
| 14      | 8                                   | 82± 5  | 86                   | 100                                | 585                                      | 207   | ---    | 146   | 101   |
| 15      | 8                                   | 100± 4                                       | 121                  | 0                                  | 191                                      | 231   | 157    | 167   | 137   |
| 55      | 8                                   | 88± 5  | 128                  | 100                                | 502                                      | 166   | 133    | ---   | 135   |
| 46      | 8                                   | 93± 7  | 140                  | 100                                | 203                                      | 270   | 265    | 266   | 272   |
| 20      | 8                                   | 108± 5                                       | 131                  | 100                                | 207                                      | 162   | 148    | 144   | 137   |
| 3       | 8                                   | 94± 5  | 119                  | 100                                | 304                                      | ---   | 225    | 265   | 153   |
| 51      | 8                                   | 93± 4  | 131                  | 100                                | 191                                      | 216   | 146    | 146   | 131   |
| 61      | 8                                   | 101± 7                                       | 148                  | 100                                | 198                                      | 171   | 151    | 149   | 139   |
| 3       | 9                                   | 94± 5  | 128                  | 0                                  | 183                                      | ---   | 480    | 136   | 131   |
| 15      | 9                                   | 100± 4                                       | 130                  | 100                                | ---                                      | 250   | ---    | 169   | 104   |
| 51      | 9                                   | 93± 4  | 126                  | 100                                | 165                                      | ---   | 144    | ---   | 166   |
| 62      | 9                                   | 90± 5  | 146                  | 100                                | 178                                      | 157   | 148    | 157   | 138   |
| 14      | 12                                  | 82± 5  | 132                  | 0                                  | 148                                      | ---   | 142    | 133   | 185   |
| 55      | 12                                  | 88± 5  | 119                  | 0                                  | 241                                      | 155   | 137    | 126   | 146   |

In order to control the results eight glucose tolerance tests were done on rats not injected with the parotid extracts. Four of these tests were done after eight daily injections of physiologic saline, and two after eight daily beef extract broth injections. Two were not injected (Table IV).

The results of the control rats closely simulated glucose tolerance curves in normal human beings. Practically all the blood sugar curves following the use of precipitate II presented a picture similar to that in diabetic patients (Table III). The one-half-hour samples of the latter ranged from 148 mg. per cent to 585 mg. per cent, whereas the one-half-hour samples of the control animals ranged from 117 mg. per cent to 153 mg. per cent. The one-hour test samples of the rats injected with the parotid precipitate ranged from 155 mg. per cent to 270 mg. per cent, whereas that of the controls ranged from 101 mg. per cent to 128 mg. per cent. In the one-and-one-half-hour samples blood sugars ranging from 133 mg. per cent to 480 mg. per cent were obtained in the injected animals, in comparison to blood sugars ranging from 90 mg. per cent to 131 mg. per cent in the control animals. All the two-hour blood sugars of the control animals were normal, whereas the two-hour samples of the injected animals ranged

from 126 mg. per cent to 266 mg. per cent. The three-hour samples from the injected animals ranged from 101 mg. per cent to 272 mg. per cent (Tables III and IV).

TABLE IV  
GLUCOSE TOLERANCE TESTS OF CONTROL RATS

| RAT NO. | MEAN OF SIX BLOOD SUGARS PRIOR TO INJECTIONS | FASTING BLOOD SUGARS | SUBSTANCE USED     | AMOUNT INJECTED (C.C.) | BLOOD SUGARS FOLLOWING INJECTION (MG. %) |        |        |        |       |
|---------|--|----------------------|--------------------|------------------------|--|--------|--------|--------|-------|
|         |  |                      |                    |                        | ½ HR.                                    | 1 HR.  | 1½ HR. | 2 HR.  | 3 HR. |
| 60      | 97± 5  | 94                   | Physiol. saline    | 3                      | 139                                      | 126    | 101    | 99     | 83    |
| 61      | 101± 7                                       | 102                  | Physiol. saline    | 3                      | 130                                      | 124    | 121    | 103    | 103   |
| 62      | 90± 5  | 115                  | Physiol. saline    | 3                      | 153                                      | 126    | 131    | 110    | 103   |
| 63      | 103± 4                                       | 90                   | Physiol. saline    | 5                      | 145                                      | 128    | 115    | 126    | 102   |
| 33      | 99± 5  | 117                  | Beef extract broth | 5                      | 139                                      | 122    | 113    | 94     | 101   |
| 4       | 91± 5  | 79                   | Beef extract broth | 5                      | 131                                      | 118    | 115    | 119    | 88    |
| 64      | 105± 5                                       | 79                   | 0                  | 0                      | 117                                      | 103    | 124    | 99     | 94    |
| 20      | 108± 5                                       | 101                  | 0                  | 0                      | 121                                      | 101    | 90     | 97     | 79    |
| Mean    | 99± 2  | 103± 5               |                    |                        | 134± 4                                   | 119± 4 | 114± 5 | 106± 4 | 95± 4 |

TABLE V  
GLUCOSE TOLERANCE TESTS OF RATS INJECTED WITH PRECIPITATE II EXCLUSIVE OF TESTS SHOWING EXTREME OR SECONDARY PEAKS

| RAT NO.                             | NO. OF CONSECUTIVE DAILY INJECTIONS | MEAN OF SIX BLOOD SUGARS PRIOR TO INJECTIONS | FASTING BLOOD SUGARS | PRECIPITATE II PRIOR TO TEST (MG.) | BLOOD SUGARS FOLLOWING INJECTION (MG. %) |         |        |         |         |
|-------------------------------------|-------------------------------------|--|----------------------|------------------------------------|--|---------|--------|---------|---------|
|                                     |                                     |  |                      |                                    | ½ HR.                                    | 1 HR.   | 1½ HR. | 2 HR.   | 3 HR.   |
| 49                                  | 1                                   | 97± 4  | 97                   | 100                                | 160                                      | 216     | 196    | 202     | 128     |
| 2                                   | 1                                   | 93± 2  | 94                   | 100                                | 189                                      | 169     | 158    | 139     | 128     |
| 14                                  | 3                                   | 82± 5  | 117                  | 100                                | 171                                      | 162     | 160    | 129     | 115     |
| 15                                  | 3                                   | 100± 4                                       | 106                  | 100                                | 153                                      | 178     | 162    | 146     | 108     |
| 20                                  | 8                                   | 108± 5                                       | 131                  | 100                                | 207                                      | 162     | 148    | 144     | 137     |
| 46                                  | 8                                   | 93± 7  | 140                  | 100                                | 203                                      | 270     | 265    | 266     | 272     |
| 15                                  | 8                                   | 100± 4                                       | 121                  | 0                                  | 191                                      | 237     | 157    | 167     | 137     |
| 61                                  | 8                                   | 101± 7                                       | 148                  | 100                                | 198                                      | 171     | 151    | 149     | 139     |
| 51                                  | 8                                   | 93± 4  | 131                  | 100                                | 191                                      | 216     | 146    | 146     | 131     |
| 15                                  | 9                                   | 108± 4                                       | 130                  | 100                                | ---                                      | 250     | ---    | 169     | 104     |
| Mean                                | -                                   | 97± 1  | 122± 6               |                                    | 185± 6                                   | 203± 13 | 171± 4 | 166± 12 | 140± 15 |
| Mean increase over control rats     |                                     |  |                      |                                    | 51± 8                                    | 84± 14  | 57± 6  | 60± 13  | 45± 18  |
| Per cent increase over control rats |                                     |  |                      |                                    | 38                                       | 71      | 50     | 57      | 47      |

Three distinct types of curves were obtained. The most frequently encountered (Table V) reached its peak within one hour, with an average 71 per cent higher than that of the controls. The blood sugars of the control animals uniformly reached a peak in one-half hour. There was a 47 per cent increase in the three-hour samples over those of the controls. The average of the three-



hour blood sugars of the test animals was higher than that of their fasting blood sugars; whereas the average of the three-hour samples of the control rats was lower than the average of their fasting samples (Tables IV and V). All the average increases in the blood sugars of the test animals were statistically significant.

The second type of curve was encountered in Rats 3, 55, and 14 after eight daily injections, and in Rat 3 after nine daily injections. Extreme blood sugar rises from 304 to 585 mg. per cent were obtained in the one-half hour to the one and one-half hour samples (Table III). Sharp drops in the following samples followed these extreme peaks. Secondary peaks in the two- or three-hour blood samples were encountered in the tolerance test of six rats.

Although the majority of the tolerance curves paralleled the first type, the rats did not react constantly with any one specific type.

3. Precipitate III. Five milligrams of precipitate III were injected into seven rats. The mean of the blood sugars one hour after injection was 57 per cent higher than that of the mean of 42 blood sugars prior to the injections, representing a highly significant increase. The mean of the three-hour blood sugars was 25 per cent higher than that of the control sugars (Table VI).

TABLE VI  
EFFECTS OF PRECIPITATE III ON BLOOD SUGARS

| RAT NO.                         | MEAN OF SIX<br>BLOOD SUGARS<br>PRIOR TO<br>INJECTIONS | AMOUNT<br>INJECTED<br>(MG.) | BLOOD SUGARS AFTER INJECTIONS<br>(MG. %) |        |
|---------------------------------|---|-----------------------------|--|--------|
|                                 |   |                             | 1 HR.                                    | 3 HR.  |
| 29                              | 93± 6   | 5                           | 176                                      | 115    |
| 52                              | 96± 2   | 5                           | 157                                      | 113    |
| 51                              | 93± 4   | 5                           | 149                                      | 124    |
| 55                              | 88± 4   | 5                           | 148                                      | 114    |
| 54                              | 94± 6   | 5                           | 138                                      | 110    |
| 15                              | 100± 4  | 5                           | 146                                      | 124    |
| 21                              | 104± 5  | 5                           | 142                                      | 134    |
| Mean                            | 96± 2   |                             | 151± 5                                   | 120± 3 |
| Mean increase                   |   |                             | 55± 5                                    | 24± 4  |
| Per cent increase over controls |   |                             | 57                                       | 25     |

TABLE VII  
GLUCOSE TOLERANCE TESTS OF RATS INJECTED WITH PRECIPITATE III

| RAT<br>NO. | NO. OF<br>CONSEC-<br>UTIVE<br>DAILY<br>INJEC-<br>TIONS | MEAN OF<br>SIX BLOOD<br>SUGARS<br>PRIOR TO<br>INJEC-<br>TIONS | FASTING<br>BLOOD<br>SUGARS | PRECIPI-<br>TATE III<br>PRIOR TO<br>TEST<br>(MG.) | BLOOD SUGARS FOLLOWING INJECTION<br>(MG. %) |       |        |       |       |
|------------|--|---|----------------------------|---|---|-------|--------|-------|-------|
|            |  |   |                            |   | ½ HR.                                       | 1 HR. | 1½ HR. | 2 HR. | 3 HR. |
| 51         | 1  | 93± 4   | 94                         | 3   | 202   | 128   | 149    | 151   | 171   |
| 61         | 1  | 101± 7  | 74                         | 3   | 128   | 128   | 151    | 185   | 106   |
| 62         | 1  | 90± 5   | 110                        | 3   | 148   | 160   | 146    | 131   | 108   |

Three glucose tolerance tests (Table VII) after injection of 3 mg. of precipitate III were similar to those obtained after the injection of 100 mg. of precipitate II.

(B) *Tissues*.—Two rats were autopsied after having received eight daily injections of beef extract broth. Seven rats were autopsied after nine to twelve daily injections of precipitate II, and in addition one injection of precipitate III.

The pancreas of the rats injected with beef extract broth presented no changes in the parenchyma of the islands of Langerhans.

The parenchyma of the pancreas of those rats which had received the injections of the several parotid extracts was normal. The majority of the islands of Langerhans were normal in size, shape, and arrangement of the island cells. In all the specimens, however, there were islands exhibiting marked granular degeneration, hydropic degeneration, disorganization of the normal island pattern, and varying degrees of infiltration with small round cells and fibroblasts. In several islands there was almost complete replacement of the island cells with young fibroblasts. Sections of the parotids, livers, spleens, kidneys, and thyroid glands of the extract injected animals revealed no pathologic changes.

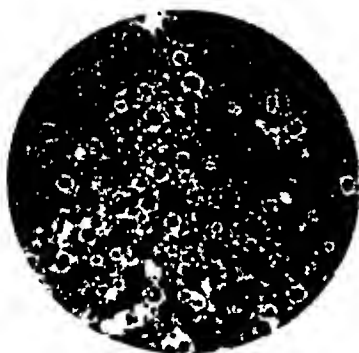


Fig. 1.

Fig. 1.—Crystals of precipitate III ( $\times 320$ ).

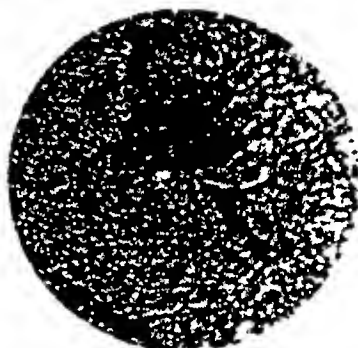


Fig. 2.

Fig. 2.—Replacement of island cells with fibroblasts ( $\times 320$ ).

#### DISCUSSION

The constant and prolonged hyperglycemias following intraperitoneal injections of the parotid extracts indicate the existence of an insulin antagonist secreted by the parotid gland. The markedly significant 84 per cent increase in the one-hour samples following the injection of precipitate I into seven fasting rats may possibly be explained by assuming that glycogenesis was being inhibited, whereas glycogenolysis was proceeding at a normal rate, thus resulting in an abnormal accumulation of endogenous glucose in the blood.

Injections of an average of only 5 mg. of precipitate III into seven rats produced an average increase of 57 per cent in the one-hour samples. Similar increases were obtained following the use of precipitate II in nine rats. Injections of 5 mg. of the refined crystalline precipitate III produced hyperglycemias comparable to those following 50 mg. of precipitates I and II (Tables I, II, and VI). We cannot, however, state that precipitate III was ten times as potent as precipitates I and II, since the end point of the inhibiting effects of the extract has not yet been determined. A chemical analysis of the crystals constituting precipitate III is still in progress.

The blood sugars of rats that received the parotid extract for the first time returned to the preinjection level within twenty-four hours, whereas the fasting blood sugars of those rats that received repeated daily injections were 31 per cent greater than their preinjection average.

These findings demonstrate that, although the inhibiting effect of the parotid substance disappears within twenty-four hours after one massive dose, repeated injections tend to maintain the fasting blood sugars at higher levels, even after the cessation of the injections due to the degenerative changes in the islands of Langerhans. This effect is exemplified by the glucose tolerance tests of Rats 15, 3, 14, and 55 (Table III), in which occurred peaks of 231, 480, 185, and 241 mg. per cent, twenty-four hours after the last injection of parotid extract. The normal glucose tolerance curves of control Rats 4 and 33, that had been repeatedly injected with beef extract broth, make improbable the possibility that a nonspecific irritating effect of intraperitoneal injections of a tissue extract may have resulted in hyperglycemia. Sections of the pancreas of these rats revealed no abnormal changes. Likewise, the normal glucose tolerance curves of the four rats which received repeated intraperitoneal injections of physiologic saline solution exclude the possibility that the hyperglycemias in the test animals were the result of repeated peritoneal punctures.

It is difficult in the present stage of this study to determine whether the degenerative changes in the islands of Langerhans following injections of the parotid extracts represent a reaction to a specific cell toxin, or are the end results of an accelerated type of atrophy of disuse following prolonged inhibition of all function.

Werh and Altshuler, and Harrow and co-workers, prepared blood-sugar raising substances from the urine of diabetic patients. The former found greater amounts of this substance in the urine of persons with diabetes than in the urine of normal individuals, and even greater amounts in urine in uncontrolled diabetes than in that of controlled diabetes. In the present study the method used by Harrow and associates to extract blood-sugar raising substances from the urine was applied in a modified form to the parotid gland. A potent blood-sugar raising extract was secured.

Himsworth<sup>16</sup> in 1936 suspected the presence of an insulin antagonist. He suggested that diabetes mellitus in the insulin-sensitive person was due to a lack of insulin, whereas diabetes in the insulin-insensitive person was due to an insulin antagonist. It is possible that his two types of patients with diabetes represent two different stages of a condition with the same etiologic mechanism. An island of Langerhans antagonist may cause primary effects through its specific insulin antagonism and secondary effects following permanent damage to the islands of Langerhans. This damage may be due to either an atrophy of disuse, or a specific cell toxin.

#### SUMMARY

1. A potent blood-sugar raising extract was prepared from the parotid gland.
2. Degenerative changes in the islands of Langerhans followed repeated injections of this extract in experimental animals.

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14 FIFTH AVENUE

## THE ORAL ASCORBIC ACID TOLERANCE TEST AND ITS APPLICATION TO SENILE AND SCHIZOPHRENIC PATIENTS\*

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IT IS well known that there is a wide difference in the amount of ascorbic acid intake necessary to prevent the outward manifestation of scurvy and the amount desirable for optimal health.<sup>1</sup> As evidence accumulates concerning the true physiologic functions of vitamin C, such as the work of Sigal and King<sup>2</sup> on vitamin C levels and resistance to bacterial toxins, and of Ecker and co-workers<sup>3</sup> on the relation between ascorbic acid and guinea pig complement, there will be an increasing need of a simple clinical procedure for determining the status of vitamin C nutrition in patients. The many techniques already available are well reviewed by authorities in this field in *The Vitamins*, edited in 1939 by the American Medical Association. Determination of the "resting level" of excretion<sup>4-6</sup> and of "saturation deficits"<sup>7, 8</sup> involve so many difficulties

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of performance and interpretation, not the least of which is the long time necessary for the test, that these techniques have not found general adoption in the clinic.

The significance of the plasma ascorbic acid content in single fasting specimens has been considered by many<sup>9-12</sup> to be of very limited value as an index of vitamin C nutrition, a belief that is further confirmed by our work. The lack of agreement<sup>13, 14</sup> concerning the clinical significance of such fasting values is itself evidence of the inadequacy of this otherwise simple test. Butler and Cushman<sup>15</sup> have found that the white blood cells and platelets may retain most of their ascorbic acid content even when the plasma ascorbic acid has been reduced to zero, and are themselves reduced to low ascorbic acid values only in acute scurvy. These authors believe that ascorbic acid analyses of these cells "provide indices of vitamin C deficiency which extend beyond the limit of the index furnished by fasting plasma concentrations." Their data indicate, however, that the vitamin C content of these cells is nearly maximal at low plasma concentrations, so that while analysis of these cells may indicate the severity of an existing scurvy, it would not be as sensitive an index of less severe vitamin C unsaturation.

Since the basic interest in "saturation tests" is the existing vitamin C content of the tissues, and since this substance appears to be in the free state both in the tissues and plasma, a reliable index should be furnished by the rate of penetration of the vitamin from the plasma into the tissues. Thus upon feeding the vitamin, if the tissue ascorbic acid content were low, the rapid flow of ascorbic acid from the plasma to such tissues should result in only a small rise of the plasma ascorbic acid; if the tissues were "saturated," then the plasma content should increase markedly. These suppositions were found to be true. Such a tolerance curve with vitamin C administered both orally and intravenously has been used in conjunction with other "saturation tests" by Portnoy and Wilkinson<sup>11</sup> in a study of normal patients and those with ulcer. The test was not studied in detail by these authors, however, and no attempt was made to relate the various degrees of saturation with the type of tolerance curve obtained. Intravenous "test dose" methods with measurement of the urinary excretion have been suggested<sup>16-19</sup> to eliminate possible errors due to differences in intestinal absorption rates. Intravenous administration must in our opinion, and in agreement with that of van Eekelen and Heinemann,<sup>20</sup> involve a sudden flooding of the kidney and loss of a large amount of ascorbic acid, so that the blood values and urinary excretions observed must be greatly influenced by the speed of injection and secretory ability of the kidney. In the "oral" test to be described, the dosage was so chosen that rarely more than 10 to 15 per cent of the total dose was excreted during the five-hour test. Hence the principal factor in determining differences in the curves appeared to be the fundamental one, namely, the rate of penetration of the vitamin from the plasma into the tissues. The shape of an individual curve is, of course, determined in large part by the rate of intestinal absorption, as is the case with a sugar tolerance curve, but in our series we have not encountered any cases in which this factor appeared to be the main determinant of differences in the shape of a group of oral tolerance curves. Proof of this fact lies chiefly in the satisfactory explanation of differ-

ences on the basis of vitamin C nutrition alone, and on those changes produced by experimental feeding and deprivation of the vitamin in a single individual, where the factor of gastrointestinal absorption must be considered to remain constant. If cases of exceptionally slow absorption were encountered employing this test, the curve would at least suggest deficiency and lead to vitamin C therapy, and if this failed to produce a "saturated" type of curve, other methods of administration for therapy would be indicated.

This paper includes a study of (a) the use of the oral ascorbic acid tolerance test for a rapid clinical diagnosis of the degree of subclinical vitamin C deficiency; (b) the changes produced in the curve upon experimentally saturating and depleting the same individual of vitamin C; (c) the analysis of sufficient tolerance curves to distinguish at least four "types" characteristic of various degrees of vitamin C nutrition; and (d) the application of the method to a study of schizophrenic and senile patients.

#### METHOD

The test is best performed in the morning. A sample of blood (approximately 7 c.c.) from a fasting patient is taken with a dry syringe and ejected into a dry pyrex tube containing approximately 20 mg. of potassium oxalate as an anticoagulant. Mixing is accomplished by gentle inversions rather than by vigorous shaking to avoid hemolysis. A dose of 6.0 mg. of ascorbic acid per kilogram of body weight is dissolved (immediately before feeding) in one-half glass water and fed, the time in minutes being noted. Since no food should be given for the following five hours, we have allowed, after the vitamin C, a breakfast consisting of a cup of coffee and plain toast. This did not materially affect the resultant curve, but made the test more tolerable to the patient. Although in our experimental work we have taken five more specimens of blood to determine the details of the curve, sufficient information for clinical tests may be obtained by taking blood specimens at only the two-and-one-half- and five-hour points. The blood samples should be chilled immediately after drawing until they are analyzed (the same day). The chemical determinations of plasma ascorbic acid were done by a method developed in this laboratory<sup>21</sup> which proved to be a rapid clinical method for serial analysis. It is a modification of the Mindlin and Butler procedure,<sup>22</sup> with extraction of the unchanged 2,6 dichlorophenolindophenol into xylene, which insures its stability.

The values obtained in milligrams per cent are plotted against time in minutes.

#### INTERPRETATION OF CURVES

For the sake of clarity of presentation and economy of space it would seem better to present first a chart summarizing the types of curves which have been distinguished in our group of 60 such determinations, and to discuss the bulk of our data by reference to such a chart. Special illustrative curves will be presented after the generalized data. Fig. 1 shows a "mapping out" of convenient areas (outlined by dark lines) into which nearly all individual curves can be allocated.

These areas have been designated tentatively by the terms "saturated," "low normal," "high normal," and "undersaturated," but the distribution of subjects within these groups must vary somewhat according to location and

dietary habits. In view of such experiments as that reported by Crandon and Lund,<sup>23</sup> in which the plasma ascorbic acid was reduced (experimentally) to zero without clinical signs of scurvy or other pathology, it is in fact difficult at the present time to define sufficiency and deficiency with respect to vitamin C. Since in clinical scurvy the plasma ascorbic acid may not reach the zero level,<sup>14</sup> the clinical syndrome probably also depends on the intake of other dietary factors, and it becomes desirable to be able to detect milder degrees of ascorbic acid deficiency.

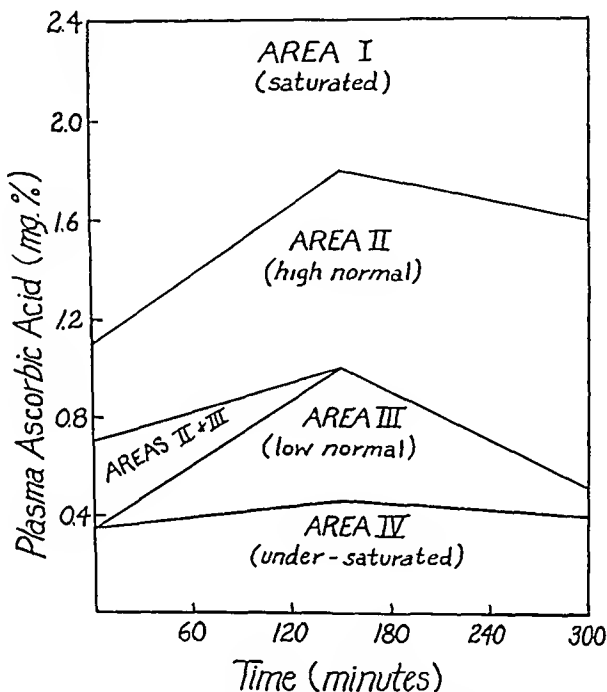


Fig. 1.—Areas chosen to classify types of tolerance curves obtained.

In Fig. 1 it may be noted that there is a small area common to both Areas II and III. This will be discussed later, but it signifies that in spite of a fasting value as low as 0.4 mg. per cent, further characteristics of the curve may, nevertheless, indicate that it should be placed in Area II.

Fig. 2 illustrates some typical curves obtained and their proper allocation in the defined areas which are in this figure represented by dotted lines.

*Type I* curves, as illustrated, are the type given by "saturated" patients. This type has been encountered only twice among our group of patients, but can be produced at will by feeding extra vitamin C, e.g., 200 to 300 mg. per day for two weeks. In 15 persons possessing among themselves all the other types of curves, we have, by feeding ascorbic acid, converted them into "saturated" persons possessing the type I curve. A typical curve is characterized by a high initial ascorbic acid value, a moderate rise, and generally a failure to decline again in the experimental period. The failure to rise to higher levels is considered to be due to exceeding the kidney threshold (assumed by many<sup>24</sup> to be approximately 1.4 mg. per cent) so that the rate of excretion is equal to or exceeds the rate of intestinal absorption. Thus subjects showing this type of

curve usually excrete in the urine large proportions (60 to 100 mg.) of the test dose. In addition, "saturation" of the tissues prevents these tissues from absorbing any considerable amount of plasma ascorbic acid.

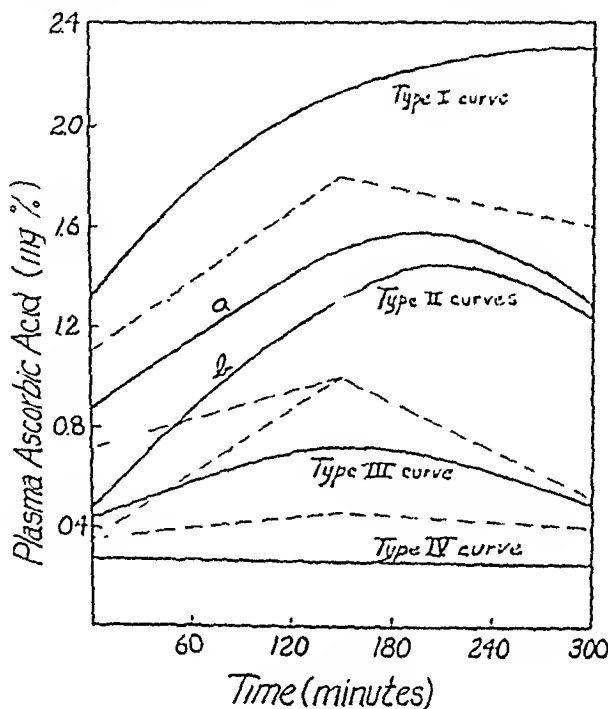


Fig. 2.—Illustrative oral ascorbic acid tolerance curves.

*Type II* curves, as illustrated in Fig. 2, were found in testing 13 normal nurses (male and female) and 20 hospital patients. All of these had normal dietary habits, being on the regular hospital diet. This group displays many variations in the tolerance curve, but all are characterized by marked rises and various rates of fall. The rise signifies that the rate of intestinal absorption exceeds the rate at which the relatively saturated tissues can absorb ascorbic acid from the plasma, the latter rate displaying itself only after intestinal absorption is more or less complete (latter part of curves). Although urinary excretion (from 15 to 50 mg. during the five hours) parallels the height of the curve in many instances, there were several cases of abnormally higher or lower excretions than that predicted from the curves.

Several persons possessing type II curves could not have been distinguished from patients possessing curves in the next lower state of saturation on the basis of fasting values alone (see type II-b). Thus in our group of 33 type II curves there were 9 subjects whose fasting values fell between 0.35 and 0.7 mg. per cent but who could not be considered from the tolerance curves to be any less "saturated" than those possessing higher fasting values.

*Type III* curves have been found in four patients of our series and in two other persons during a period of experimental deprivation of vitamin C. The type is characterized by relatively low fasting values (0.35 to 0.7 mg. per cent), with only slight or very transitory rises in the tolerance curve. It is included



as a category, since it is probable that this intermediate type represents a relative unsaturation and will occur quite frequently in the general clinic where patients of more varied dietary habits are encountered.

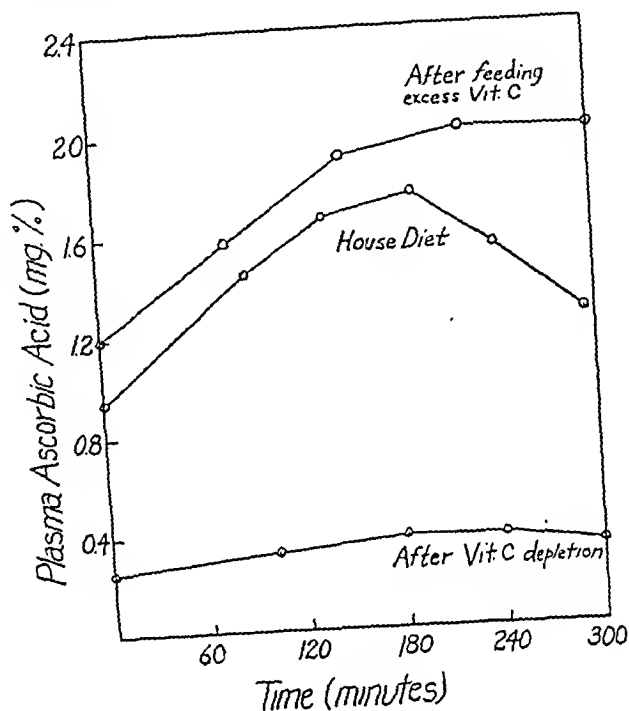


Fig. 3.—Experimental changes produced in tolerance curves of patient N by feeding and deprivation of vitamin C.

*Type IV*, illustrated in Fig. 2, is typical of vitamin C deficiency. Five of our group, all senile patients, have shown such curves and will be discussed in more detail later. In five experimental persons placed on a vitamin C deficient diet, we have likewise obtained such curves. In such cases the flow of ascorbic acid from the plasma to the unsaturated tissues apparently always exceeds the rate of intestinal absorption. Urinary excretion during the period never exceeds 2 to 3 mg. in these cases. Such curves may not be uncommon in the general population and certainly represent a state of unsaturation requiring vitamin C therapy.

#### EXPERIMENTAL CHANGES IN THE TOLERANCE CURVE

In order to demonstrate experimentally the variations in the tolerance curve of the same patient on different vitamin C intakes, the tolerance curves of three persons (schizophrenic) were determined on the regular house diet after two weeks of daily administration of 200 mg. vitamin C, followed by seven weeks on a vitamin C deficient diet. Two more patients were placed on the deficient diet. As illustrative of the data obtained, the tolerance curves of one of the first group are shown in Fig. 3, the other data are recorded in Table I. In each case we were able, by feeding ascorbic acid, to produce a tolerance curve, indicating greater saturation, and by deprivation to produce an "unsaturated"

type of curve. Since none of these patients or any others showing type IV curves ever showed any signs of clinical scurvy, the oral tolerance test described here is obviously to distinguish relative degrees of saturation or to differentiate between sufficiency and mild deficiencies.

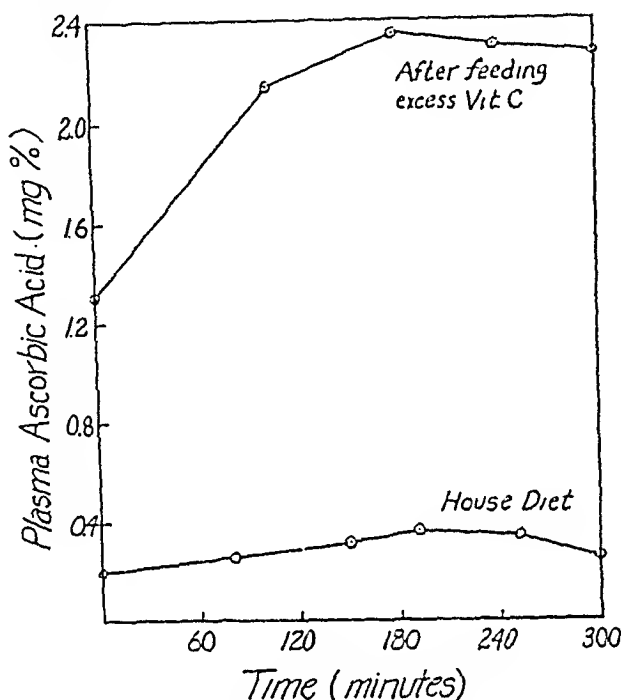


Fig. 4.—Oral tolerance curves on senile patients, before and after supplementary vitamin C feeding.

#### VITAMIN C AND SENILITY

Other workers<sup>25-27</sup> have given evidence to indicate a vitamin C lack in senility. Although Berkenau<sup>27</sup> has seemed to establish that these findings indicate a true hypovitaminosis C, since such patients required a longer time to "saturate," it was of interest to study such patients with our technique. We have, therefore, carried out the oral ascorbic acid tolerance test on a group of six senile persons. All showed the "unsaturated" or "low normal" (type IV or III) curves. Upon feeding 300 mg. of ascorbic acid per day for two weeks, they showed tolerance curves of type II or I, i.e., curves typical of greater saturation. One example of this is shown in Fig. 4, and the rest of the data are shown in Table II. These patients had been in the hospital on an adequate intake of vitamin C so that the deficiencies indicated by the initial tolerance curves could not be justified on the basis of dietary habits.

No satisfactory explanation (based on experimental proof) of this "endogenous" deficiency of vitamin C in senility has yet been offered.<sup>25, 28</sup> Our data indicate that the fault does not concern intestinal absorption, since such patients display typical saturation curves after feeding of supplementary vitamin C. The basis of this deficiency is under investigation. In the meantime it seems established that better degrees of saturation can be and should

be maintained in senile patients by oral administration of extra ascorbic acid. That this deficiency may be associated with the pathology of senility rather than with "old age" as such is indicated by the finding that 14 other hospital patients from ages 65 to 90 years showed fasting plasma values over 0.7 mg. per cent.

TABLE I

EXPERIMENTAL CHANGES PRODUCED IN TOLERANCE CURVES BY FEEDING AND DEPRIVATION OF ASCORBIC ACID

| PATIENT AND DIET | PLASMA ASCORBIC ACID (MG. %) |         |          |          |          | TYPE CURVE |
|------------------|------------------------------|---------|----------|----------|----------|------------|
|                  | 0 MIN.                       | 90 MIN. | 150 MIN. | 240 MIN. | 300 MIN. |            |
| K—House          | 0.75                         | 1.03    | 1.45     | 1.01     | 0.84     | II         |
| K—High vitamin C | 1.30                         | 2.02    | 2.05     | 2.01     | 1.95     | I          |
| K—Low vit. C     | 0.31                         | 0.38    | 0.44     | 0.47     | 0.42     | IV         |
| W—House          | 0.97                         | 1.52    | 1.70     | 1.85     | 1.74     | I          |
| W—High vitamin C | 1.08                         | 1.55    | 1.77     | 2.05     | 2.16     | I          |
| W—Low vitamin C  | 0.33                         | 0.34    | 0.36     | 0.41     | 0.36     | IV         |
| B—House          | 0.88                         | 1.44    | 1.57     | 1.54     | 1.37     | II         |
| B—Low vitamin C  | 0.22                         | 0.30    | 0.36     | 0.40     | 0.36     | IV         |
| S—House          | 0.62                         | 1.12    | 1.24     | 1.10     | 0.93     | II         |
| S—Low vitamin C  | 0.30                         | 0.40    | 0.42     | 0.39     | 0.36     | IV         |

TABLE II

ORAL TOLERANCE CURVES ON SENILE PATIENTS BEFORE AND AFTER SUPPLEMENTARY VITAMIN C

| PATIENT AND DIET | PLASMA ASCORBIC ACID (MG. %) |         |          |          |          | TYPE CURVE |
|------------------|------------------------------|---------|----------|----------|----------|------------|
|                  | 0 MIN.                       | 90 MIN. | 150 MIN. | 240 MIN. | 300 MIN. |            |
| F—House          | 0.26                         | 0.25    | 0.29     | 0.33     | 0.37     | IV         |
| F—High vitamin C | 1.11                         | 1.36    | 1.56     | 1.76     | 1.78     | II         |
| C—House          | 0.28                         | 0.29    | 0.27     | 0.27     | 0.28     | IV         |
| C—High vitamin C | 0.86                         | 1.17    | 1.46     | 1.66     | 1.55     | II         |
| W—House          | 0.24                         | 0.38    | 0.58     | 0.54     | 0.47     | III        |
| W—High vitamin C | 1.21                         | 1.76    | 1.83     | 1.86     | 1.84     | I          |
| R—House          | 0.34                         | 0.38    | 0.44     | 0.48     | 0.46     | IV         |
| R—High vitamin C | 0.41                         | 0.68    | 1.09     | 1.07     | 1.00     | II         |
| A—House          | 0.40                         | 0.46    | 0.47     | 0.54     | 0.60     | III        |
| A—High vitamin C | 0.69                         | 1.12    | 1.22     | 1.19     | 1.15     | II         |

In our group of 20 schizophrenic patients there have been no indications of vitamin C deficiency.

## SUMMARY

An oral ascorbic acid tolerance test has been developed to indicate the relative saturation of vitamin C in human beings. On the basis of 60 such determinations, and on experimental changes produced in the curve upon supplementary feeding and deprivation of vitamin C, four categories of relative saturation have been suggested. Since patients showing the lowest "saturation" that can be demonstrated by this test do not show any signs of clinical scurvy, the test is obviously to detect lesser degrees of ascorbic acid deficiency. The main characteristics of the tolerance curves can be adequately distinguished by a fasting blood specimen and two other specimens in the following five hours. The use of this test seems to overcome the unreliability of a single fasting determination of plasma ascorbic acid, and to eliminate the technical difficulties of urine collection involved in previously suggested tests for the degree of vitamin C saturation.

Application of this test has demonstrated a vitamin C deficiency in senile patients which is not related to dietary habits, and has indicated no disturbance of vitamin C saturation in schizophrenia.

We wish to express our thanks to Doctors Kenneth J. Tillotson and W. Franklin Wood of McLean Hospital, and to Professor A. Baird Hastings of the Harvard Medical School, for their suggestions and criticism in this work.

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# THE EFFECT OF YEAST AND MUSCLE ADENYLIC ACID IN MALNOURISHED PERSONS WITH PELLAGRA AND PERIPHERAL NEURITIS\*

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## INTRODUCTION

YEAST and crude liver extracts are effective in the treatment of pellagra beriberi, and riboflavin deficiency because they are excellent sources of the vitamins of the B complex, particularly nicotinic acid, thiamin, and riboflavin. Many less well-known compounds are also present in high concentration and, like nicotinic acid, thiamin, and riboflavin, are components of organic catalysts essential for cell respiration. Adenylic acid is one of these compounds. It is the precursor of adenylyl pyrophosphate (eophosphorylase) and is a component of a number of polynucleotides, including the nucleic acids, the coenzymes I and II, the prosthetic groups of xanthine oxidase, d-amino acid oxidase, and the yellow enzyme. These considerations suggest a relation of adenylic acid and the vitamins of the B complex, and indicate its great theoretical importance to cellular respiration and nutrition.

In this study 29 selected patients with nutritional deficiency disease were given adenylic acid prepared either from yeast or from muscle.† The patients have been divided into four groups to facilitate description of the therapeutic results.

*Group I. Sixteen pellagrins in relapse.* This group consisted of 16 persons, all of whom in previous years had responded to nicotinic acid therapy for pellagra. They had continued to subsist on deficient diets, and in the spring of 1940 again had developed clinical evidence of acute pellagra in relapse. Six of this group had multiple ulcers, extending deep in the tongue or buccal mucous membranes. The fiery red borders of the ulcerations were swollen and sharply demarcated from the surrounding tissue. The craters of the ulcers were covered with grayish-white membranes. Each of these 6 patients complained of severe pain around the areas of ulceration and of generalized burning sensations of the entire mouth and tongue. The remaining 10 patients had no ulcerative lesions of the tongue or oral mucous membranes, but each had characteristic pellagrous glossitis and complained of burning sensations of the mouth and tongue; two of these 10 patients also had riboflavin deficiency, as evidenced by severe conjunc-

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†Yeast adenylic acid was furnished through the courtesy of Merck and Co., Inc., Rahway, N. J., the Fleischmann Laboratories, New York, N. Y., and Anheuser-Busch, Inc., St. Louis, Mo. Muscle adenylic acid was supplied by the Ernst Bischoff Company, Ivoryton, Conn.

tival and circumcorneal injection, and cheilosis. None of these lesions improved while the patients were restricted to their usual deficient diet for two weeks of preliminary observation. At the end of this period the administration of adenylic acid was begun. Each person was given intravenously 50 mg. of either yeast adenylic acid or muscle adenylic acid, dissolved in sterile physiologic solution of sodium chloride, twice daily for from one to three days. The solution was injected not faster than 2 mg. per minute. The 14 patients receiving yeast adenylic acid and the 2 receiving muscle adenylic acid reported improvement in strength, and then rapid disappearance of all burning sensations of the mucous membranes within six hours. The ulcerations in the mouths of 6 pellagrins healed within two to five days after the first administration of adenylic acid. Those patients who continued to eat an inadequate diet had recurrences of the ulcerations and a diminution of strength within three to five weeks. When relapse occurred, some of these patients requested more of the "same medicine," despite the discomfort that accompanied the injection. The fiery red color of pellagrous glossitis and the cheilosis and conjunctivitis of riboflavin deficiency were not affected by adenylic acid.

*Group II. Four persons with prodromal symptoms of pellagra.* Four persons, each of whom had been observed with clinical pellagra in previous years, had continued to eat an inadequate diet. When seen in the Nutrition Clinic this year, they complained of weakness, vague pains, irritability, insomnia, and burning sensations of the skin and mucous membranes. Three were given 50 mg. of yeast adenylic acid b.i.d. and one was given 50 mg. b.i.d. of muscle adenylic acid for from one to three days in the manner described.

Within six hours after the first injection these patients reported an increase in strength and vigor, and disappearance of all burning sensations of the skin and mucous membranes. The dramatic disappearance of anxiety, irritability, and nervousness, which follows the intravenous injection of large amounts of thiamin hydrochloride, was not observed after the injection of adenylic acid in the doses used. Accordingly, after this study a single dose of 100 mg. of thiamin hydrochloride was given intravenously and was followed by relief from these nervous symptoms within a few hours.

*Group III. Six persons with peripheral neuritis which was refractory to treatment with thiamin hydrochloride and brewers' yeast.* Six patients were selected because of severe refractory peripheral neuritis. Five of these persons were addicted to alcohol; the sixth had had recurrent attacks of pellagrous glossitis for ten years and peripheral neuritis for three years because of faulty gastrointestinal absorption. A number of observers had studied these patients clinically, and had treated the peripheral neuritis for from one to three years. The neuritis had failed to respond to intensive treatment with brewers' yeast for two months or more, or to the intravenous administration of from 50 to 100 mg. thiamin hydrochloride daily for a minimum of fourteen days. Before adenylic acid was administered, neurologic examination of each person showed the following similar abnormalities which had been observed before treatment with yeast and thiamin hydrochloride: Each had severe paresthesias of the feet and legs, both spontaneous and in response to pinprick and stroke, diminished perception of

light touch and vibration in the ankles and knees, and diminished appreciation of the position of the toes. Ankle jerks were absent and knee jerks were hypoactive in every case. Each of these patients received, intravenously, 25 mg. of yeast adenylic acid twice a day for ten to thirty days.

Spontaneous paresthesias temporarily disappeared during the administration of adenylic acid. In one case, for example, hyperesthesia, located at the knee before injection, was found near the ankle during the injection, but within fifteen minutes after the termination of the injection the level was found at the knee again. Similar responses were observed in each patient following each injection, but there was also a gradual and steady decrease in all painful sensations in the legs. Within ten to twenty-one days spontaneous pain and demonstrable hyperesthesia disappeared, perception of light touch improved, and the patients were able to walk without pain, whereas before treatment they could not bear the weight of bedclothes on their feet and legs. In 3 cases the knee jerks changed from hypoactive to active during the period of treatment, and the perception of vibration, previously absent in the knees, returned. Ankle jerks, the perception of vibration over the malleoli, and appreciation of the position of the toes, did not change during the period of observation.

Electrocardiographic tracings taken on these 6 persons, which were normal before injection, showed the following temporary changes during the injection: Tachycardia in 3 persons; P-R interval increases of more than 0.02 second in 4; Q-T interval increases of more than 0.02 second in 2; inverted cone-shaped T-waves in all leads in 1; inverted P-waves in Lead 3 in 1; flattened T-waves in all leads in 3. These alterations disappeared within five minutes after the cessation of the injection.

*Group IV. Three persons with ulcerations of the tongue whose diets were adequate and who had no evidence of nutritional deficiency.* Three persons were selected for comparison with the patients in group I because of recurrent superficial ulcerative lesions of the tongue for which no cause could be found. Their diets were adequate, they presented no evidence of nutritional deficiency disease, and the appearance of the ulcers bore no relationship to the season of the year. Each of these persons was injected intravenously with 50 mg. of yeast adenylic acid twice a day for from three to five days. At the time of injection there was no change in their sense of well-being and later no change in the appearance of the ulcers. They were given 500 mg. of nicotinic acid by mouth for one week. Still there was no healing in the ulceration or improvement in the burning sensations of the tongue in the regions of ulceration.

#### REACTIONS OBSERVED DURING THE INJECTION OF YEAST AND MUSCLE ADENYLIC ACID

Immediate reactions of varying degrees of severity, such as were described in normal persons,<sup>1</sup> were invariably observed with both the yeast and muscle adenylic acid preparations. Rapid injections of 5 to 10 mg. of either compound produced, within twenty seconds, a flushing of the face, deep inspiration, transient tachycardia, sensation of suffocation, and impending disaster; whereas slow injection of 2.0 mg. per minute produced mild hyperpnea, tachycardia, abdominal cramps, tingling sensations in the mouth, and mild apprehension.

Patients who had burning sensations in the abdomen and about the eyes and mouth stated that these sensations increased temporarily during the period of injection. Transient increase in lacerimation and injection of the conjunctival vessels were noted in several cases, particularly in patients who had conjunctival congestion. In contrast, the intravenous injection of adenine sulfate had no pharmacologic effect and no apparent physiologic effect.

These observations on the immediate reaction and the electrocardiographic alteration during the injection of adenylic acid support the previous observation of Honey, Ritchie, and Thomson,<sup>2</sup> Rothmann,<sup>3</sup> and Ruskin and Katz,<sup>4</sup> on the effect in human beings of intravenous and intramuscular injections of adenosine and ferrous adenyate, substances which act like adenylic acid in all particulars which we have studied.

#### SUMMARY AND CONCLUSIONS

1. These observations and those previously made suggest that the role of adenylic acid in human nutrition is important and extremely diversified. Within from two to five days following the daily intravenous administration of 50 mg. of adenylic acid from yeast or from muscle, the ulcers in the mouths of 6 malnourished persons disappeared. In contrast, the ulcerations in the mouths of 3 persons who had stomatitis, but no evidence of dietary deficiency, did not improve following the injection of similar amounts of adenylic acid. The administration of either yeast adenylic acid or muscle adenylic acid to persons with pellagrous glossitis or persons with subclinical symptoms of pellagra was followed by rapid improvement in strength and well-being and disappearance of the burning sensations of the mucous membranes.

2. The intravenous administration of yeast adenylic acid to 6 persons with peripheral neuritis who had failed to respond to brewers' yeast or to massive doses of thiamin hydrochloride, intravenously, was followed by striking clinical changes after a period of from ten days to three weeks. Spontaneous pain and demonstrable hyperesthesia disappeared, perception of light touch improved, and the patients were again able to walk without pain.

3. The intravenous administration of either muscle adenylic acid or yeast adenylic acid produced similar immediate reactions. Because of these reactions and the concomitant electrocardiographic changes, we do not recommend adenylic acid for general therapeutic use. Nicotinic acid and thiamin hydrochloride, given in conjunction with a diet rich in protein and calories, continue to be the most efficient therapeutic measures for most malnourished persons with ulcerative stomatitis and peripheral neuritis.

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# LABORATORY METHODS

## GENERAL

### FLUORESCENT STAINING TECHNIQUE FOR DETECTION OF ACID-FAST BACILLI\*

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ACID-FAST staining by the Ziehl-Neelsen method is a classic procedure. There are very few laboratories, either private, institutional, municipal, or state, that do not use this method. In view of the difficulties encountered with the Ziehl-Neelsen method, the introduction of a cold stain, followed simply by a decolorizing solution, is appealing in itself. Such is the procedure of the fluorescent technique.

The remarkable simplicity of the technique and the excellent results reported by Richards and Miller of New York prompted us in the Chicago Branch Laboratory of the Illinois State Health Department to apply it to use in a busy routine diagnostic laboratory under normal conditions.

A demonstration by Dr. Edmund Kline at the American Public Health Association Meeting in Detroit in October, 1940, was indeed convincing. It was not until January that we were able to experiment with this technique on a large scale, at which time accessory equipment was made available to us through the courtesy of the Spencer Lens Company.

The application of fluorescence microscopy to acid-fast bacteria and the use of filament rather than arc lamps are due to two German physicians, Hagemann and Keller. The equipment at present makes the method available for use with any monocular microscope and may be used only with materials whose fluorescence will be excited by the available ultraviolet light and the fluorescence that will pass through a yellow filter to the eye.

During the process of investigation of this method with direct and concentrated specimens, the following claims were kept in mind: (1) An increase in positives. (2) Reduction in fatigue in spite of the use of a monocular microscope. (3) Absence of interfering fluorescing substances. (4) Low cost of initial and upkeep of accessory equipment. (5) Rapidity of the procedure. (6) Practicability in small and large laboratories, hospital, and health department. (7) No specific experience necessary. During the course of this paper I shall try to clarify these claims.

\*From the Illinois State Health Department, Chicago.

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The method and equipment were developed and tested by Dr. Oscar W. Richards, of the Spencer Lens Company Research Department, and were first applied at the Meyer Memorial Hospital in Buffalo by Dr. D. K. Miller. Eleven sputa in their first series of 100 were found positive with this method which were negative with Ziehl-Neelsen. The fluorescence technique on direct smears had greater sensitivity than on concentrations with Ziehl-Neelsen. Acid-fast bacilli found in a spinal fluid and pus were not demonstrated by Ziehl-Neelsen. They also mentioned that one-third less time is required for preparing and reading smears.

Let us take up the necessary accessory equipment and procedure of the technique. (1) A monocular microscope is necessary. The present source of ultraviolet light is limited so that it would be almost impossible to obtain satisfactory light through a double body tube of a binocular microscope. We understand that this limitation is being worked on by Dr. Richards.\* The draw tube on a monocular microscope may be substituted for the binocular draw tubes of a Spencer microscope. (2) The microscope should be equipped with an 8 mm. objective and a 20 $\times$  ocular or 1.8 mm. objective and a 6 $\times$  ocular. (3) A source of light which has low voltage and high amperage, such as a Universal microscope lamp of 6.5 volts and 2.75 amperes with clear bulb and transformer, is desirable. Universal lamps Nos. 351 and 353 are suitable because the high amperage and concentrated filament furnish sufficient ultraviolet radiation for use with the monocular microscope. Apparently the less intense low voltage lamps and even many of the larger conventional lamps are not satisfactory because either less ultraviolet light is produced or too much of it is absorbed by the lenses. (4) A blue ultraviolet transmitting filter placed in front of the light source is essential. (5) An aluminum-surfaced mirror sets in front of the microscope mirror. This is necessary, since a silver mirror absorbs the ultraviolet radiation instead of reflecting it. (6) Finally a yellow light filter is placed in the ocular of the microscope. This is a complementary filter necessary to see the acid-fast bacteria which fluoresce to appear yellow.

The staining solution is composed of 0.1 Gm. of Auramine O (National Aniline Company), 3 c.c. of liquefied phenol, and 97 c.c. of distilled water. The decolorizing solution is composed of 100 c.c. of 70 per cent alcohol, 0.5 c.c. of concentrated hydrochloric acid, and 0.5 Gm. of sodium chloride. There is no counterstain.

We know that certain microorganisms are called acid-fast bacteria because they retain the basic dye (whether it be fuchsin or auramine) in a phenol or carbolic acid solution after the remaining material on the slide is made void of color with acid alcohol. The acid-fast bacteria retain auramine, a fluorescent dye, after the surrounding material has been destained with acid alcohol. When they are lighted with ultraviolet radiation, this light is absorbed and reradiated as visible yellow light. Consequently, the bacteria appear as bright self-luminous bodies on a dark, almost black, background. This effect is obtained by the blue ultraviolet transmitting filter on or in front of the light source and a yellow filter in the eyepiece. This arrangement becomes black to the eye because the

\*Personal communication.

yellow filter absorbs the radiation passed by the blue filter. (Since silver absorbs ultraviolet radiation, an aluminized mirror is substituted.) The yellow light, emitted by the organisms, since auramine is retained, passes through the eye filter and appears yellow.

Smears are made as usual and fixed by heat, as in the Ziehl-Neelsen method. Auramine is applied for two minutes, washed, and decolorized with acid alcohol for two to four minutes, and then redecolorized in a fresh solution of acid alcohol for at least two minutes, again washed and dried; it is now ready for examination. For staining purposes Coplin jars or individual staining may be used. The latter method is to be preferred. Regardless of which method may be used, it is important to use a clean, fresh solution of acid alcohol as a final decolorizer to insure complete decolorization. There is no danger of over-decolorizing, but there is trouble when insufficiently decolorized.

The matter of focusing may cause a little difficulty at first, but if maximum light is obtained before examination is attempted, this difficulty will be minimized. Place the blue ultraviolet filter on the lamp in front of the light source and focus it onto the center of the mirror. Remove ocular and focus objective as near to the stage as possible; then by adjusting the mirror so that the maximum light shines through the body tube, the slide may easily be focused.

The procedure recommended by Dr. Richards follows that an objective of 8 mm. and a 20 $\times$  ocular giving a magnification of 400 be used, thus allowing an area 20 times that of oil-immersion lens objective with 10 $\times$  ocular used ordinarily with the Ziehl-Neelsen method.

The acid-fast bacilli appear as small bright yellow bacilli against a dark background. In a pure culture of tuberculosis absolutely nothing but the organisms appear, the background being nearly black. With smears, direct and concentrated, the degree of various other substances present in sputum fluorescing varies, depending upon the method used in concentrating and any other phase used in the process of preparing the sputum for examination.

#### EXPERIMENTAL

The method and the equipment recommended have been tested under conditions of a public health department laboratory in which nearly all specimens of sputum for the examination of tubercle bacilli are mailed to the laboratory. A few specimens submitted for pneumonia typing were included in this series as direct smears. The test run included 1,123 concentrated specimens and 98 direct smears with the fluorescence method and with the Ziehl-Neelsen technique. Sputum submitted to our laboratory arrives in cresol; it is autoclaved and then centrifuged. Smears made from the sediment are stained and examined for tubercle bacilli. In addition to the recommended use of the low objective and the high ocular lens various combinations, including the oil-immersion and low ocular, were tried.

#### RESULTS

As seen in Table I a total of 1,221 specimens of sputa were tested. Of these only 98 were examined by direct smear. Ninety-six were negative and two were positive by each method. The bulk of the specimens examined under con-

ditions of concentration with cresol, autoclaving, and centrifuging did not reveal such striking differences as did the comparisons reported by Doctors Richards and Miller. Of the 1,123 specimens examined above, 998 were negative and 106 were positive by both methods. However, there were twelve specimens positive by the fluorescence method which were negative by the Ziehl-Neelsen technique (a re-examination of one of these specimens by the Ziehl-Neelsen was found to be positive). Very interesting to us, but slightly disturbing, was the fact that in seven sputa positive results were obtained by the Ziehl-Neelsen technique but could not be demonstrated by fluorescence. (Two of these smears were very thin, two were bloody, and one was unsatisfactory. Two, however, were missed completely.)

Not included in the foregoing series were four smears of pus and one spinal fluid which were negative by both techniques.

TABLE I  
SPTUM FOR TUBERCLE BACILLI

| NO. OF SPUTA | FLUORESCENCE TECHNIQUE |  | ZIEHL-NEESEN TECHNIQUE |  |
|--------------|------------------------|--|------------------------|--|
|              | DIRECT                 | CONCENTRATED*                                | DIRECT                 | CONCENTRATED                                 |
| 96           | Negative               | Negative<br>Negative<br>Positive<br>Positive | Negative               | Negative<br>Positive<br>Positive<br>Negative |
| 2            | Positive               |  | Positive               |  |
| 998          |                        |  |                        |  |
| 7†           |                        |  |                        |  |
| 106          |                        |  |                        |  |
| 12‡          |                        |  |                        |  |
| 1,221        |                        |  |                        |  |

\*Concentrated with cresol, followed by autoclaving and centrifuging.

†Two smears were very thin, two were very bloody, one smear proved to contain artifacts (acid fast), not tubercle bacilli.

‡One of these was found to be positive by Ziehl-Neelsen technique on re-examination.

NOTE: Not included in the above were 1 smears of pus and spinal fluid which were negative by both techniques.

## DISCUSSION

According to Richards and Miller, "A bright yellow tuberculosis organism is more easily seen against a dark background than the usual red stained bacterium on a blue background." As an individual, I would be inclined to agree, but I do not believe that would be the consensus of opinion of the group here. Unless there has been some experience with the dark-field microscope or some other fluorescence technique, the transformation is not as rapid and as easy as it sounds. One problem encountered is that of a mental approach. One must change his or her concept of tubercle bacilli as they should appear. The recommended use of an 8 mm. objective and 20× ocular lens gives one a wider vision and an area twenty times as large but reduces the magnification of the organisms to less than one-half. This difficulty can be overcome with practice. We would recommend that the beginner in fluorescence technique use the oil-immersion objective and a low ocular 6× or 10× just as for other stained preparations of bacteria. This would permit the eye to become accustomed to change in color contrast, without making it necessary to adjust to changes in changing the shape, size, and form of the organisms. It is true that cedar oil will fluoresce so that the background is tan rather than almost black. Also the point of focusing area or working distance is very narrow. Using the low objective

and high ocular (8 mm. and 20 $\times$ , a magnification of 400 times), one can concentrate attention on change in size and shape.

There may be claims of fatigue at first because of the dark-field and monocular scope, but if the slide is protected from the glare of surrounding light, this objection is eliminated.

The rapidity with which one can examine these slides is very striking. One-fourth to one-third the time is required with this method compared with the Ziehl-Neelsen method once the smears are made. It is true that each field covers twenty times the area of that under oil immersion and increases the speed of examination, although it is necessary to guard against the tendency to lessen the importance of adequate examination of specimen because of increased area covered.

To overcome the problem of occasional thin slides that reveal nothing and render the focusing of the field difficult, apply a spot of auramine stain on the corner of slides.

Associated fluorescence has been a decided problem and inconvenience during this experiment. The fact that cresol fluoresces and nearly all our sputa arrived in cresol reduced distinct contrast, and nearly all material in the smear fluoresced to a greater or lesser degree. This was partially overcome by increasing the time of decolorizing, using low magnification to find suspicious areas and confirming with the oil-immersion lens and 6 $\times$  ocular. The fact that cedar oil fluoresces as a dull haze blotted out the definite outlines of associated interfering fluorescing substances, while the tubercle bacilli remained clear, bright, and definite.

There has been some work by Dr. Kline\* to the effect that heating reduces the sensitivity of the fluorescence stain. This may help to explain why this series involving autoclaving did not reveal such a striking increase in the number of positives. An increase of even 1 per cent is important.

If only one microscope equipped for the fluorescent technique were available in a laboratory, only one person could look at the slides. On the other hand, several people in the laboratory could look at slides stained by Ziehl-Neelsen technique. It would appear that no time would be saved by the fluorescent method. However, this is only partially true, for by the fluorescence method one individual could look at the same number of slides that two, three, and even four, could observe by the Ziehl-Neelsen method. The accessories to this method do not render the monocular microscope unsatisfactory for routine bacteriologic examination of other smears and cultures. It becomes satisfactory for any staining use by simply changing to another ocular minus the yellow filter.

The accessories are reasonable in price. The blue ultraviolet filter, aluminum mirror, and yellow filter are available for \$10.00 from the Spencer Lens Company. A Universal light of low voltage and high amperage costs \$23.00.

Other chemicals that will fluoresce very little, or not at all, used in concentrating sputum and yet satisfactory for mailing purposes were investigated.

This investigation revealed that the fluorescent technique for acid-fast bacilli, where applied to sputum concentrated with cresol, was not more satis-

\*Unpublished work.

factory than our modification of the Ziehl-Neelsen method. It appears that cresol fluoresces to the extent of interfering with the clear fluorescence of the acid-fast bacilli.

This disadvantage does not apply to direct smears. Our series of direct smear examinations was too small to comment upon intelligently.

#### SUMMARY AND CONCLUSIONS

A total of 1,221 specimens were examined for tubercle bacilli with the fluorescent and the Ziehl-Neelsen techniques. Of these, 1,123 were specimens concentrated by cresol, autoclaving, and centrifuging. The fluorescence technique for the examination of tubercle bacilli was more sensitive than the Ziehl-Neelsen staining method, increasing positives by 1 per cent, although the use of cresol in concentration produced fluorescence which interfered with the examination of the specimens. Because of the larger areas covered and the ease of examination, the time required to examine the smears was reduced by one-fourth to one-third.

The disadvantages of a monocular microscope, the high degree of miscellaneous fluorescence, and the possible fatigue were counteracted by the advantages of larger microscopic fields, simplicity of technique, rapidity of procedure, and increase in number of positives. The simplicity of the technique permits a larger number of specimens to be handled in a shorter period of time.

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# A QUICK, EASY METHOD OF PREPARING TISSUES FOR MICROSCOPIC EXAMINATION BY COMBINING THE TECHNIQUES OF NELSON AND TERRY\*

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THE purpose of this paper is to report a quick, easy, and inexpensive method of fixing, embedding (blocking), cutting, and staining tissues for microscopic examination. The technique is simple and can be mastered by any office or laboratory assistant in a few hours. With the equipment at hand and a little experience the actual working time necessary for carrying out all technical procedures is less than ten minutes. The only time-consuming part of the method is that required for adequate hardening of the tissue block, but this demands no attention. Hardening sufficient for cutting sections occurs in from two to eight hours, depending on the temperature of the media. Cutting, staining, and mounting for immediate microscopic examination can be accomplished within a few minutes.

I claim no credit for the development of any item in this technique. In fact, the procedure described herein merely represents the manner in which I have conveniently utilized, with minor modifications, the following well-developed techniques—(1) The use of protein for embedding small tissues, developed by I. A. Nelson<sup>1</sup> in 1934. (2) A new principle in the microscopic examination of fresh unfixed tissues, developed by Benjamin T. Terry<sup>2</sup> and first reported in 1926. Terry<sup>3, 4</sup> has since reported numerous improvements in technique.

## EQUIPMENT

1. Egg albumen crystals, 4 ounces. Albumin crystals make much better blocks than does powdered egg albumen. Domestic egg albumen crystals are superior to imported preparations of the same. These can be purchased at any baker's or candy maker's supply house.†

2. A sheet of ordinary wrapping cellophane (not waterproof) or patapar parchment paper‡ for making embedding boxes. Either of these can be purchased at most department stores. Patapar is easier to handle and makes a more stable box.

3. A small dish, about the size of a one-ounce ointment jar, is satisfactory.

4. Terry's safety razor blade microtome. Directions for making this inexpensive, clever little apparatus have been published by Terry.<sup>4</sup> In a forth-

\*From the Department of Gynecological Pathology, University of Oregon Medical School. Received for publication, May 13, 1941.

†I obtained this material from Gray and Company, 1305 N. W. Davis Street, Portland, Ore.

‡Manufactured by the Patterson Parchment Paper Company, Bristol, Pa.

coming publication Terry will describe his safety razor blade "pocket" microtome. A sharp old-fashioned razor may be used for sectioning, but obviously it is less convenient.

5. Formalin, 40 per cent (neutral or slightly alkaline), 4 ounces.

6. A medicine dropper.

7. Fine-pointed thumb forceps.

8. Clean cover slips and a microconcavity slide or a special slide such as described by Terry.<sup>4</sup>

9. A small slice of silk sponge strapped to a glass slide with waterproof adhesive tape.

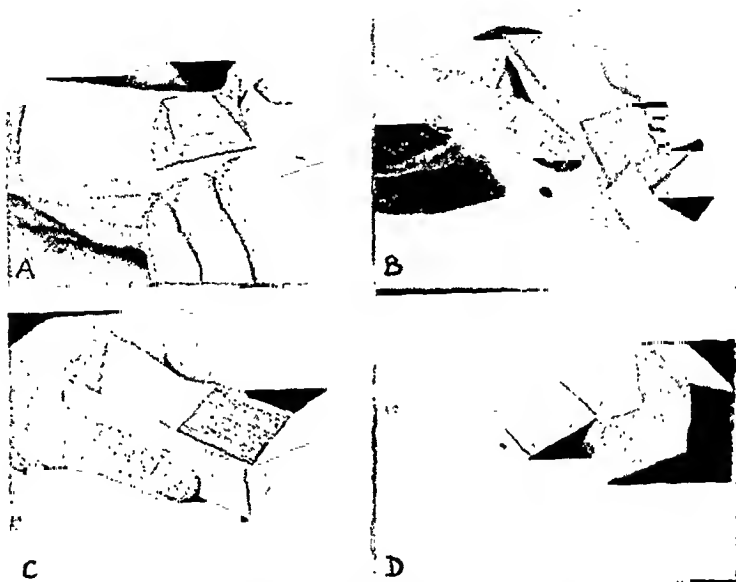


Fig. 1.—An easy method of constructing cubical boxes for embedding purposes. The wood block shown above measures  $\frac{3}{4}$  inch in all diameters.

10. A fine-pointed camel's-hair brush or a small inexpensive atomizer for applying stain.

11. Neutralized polychrome methylene blue (Terry)\* 0.5 Gm. Place this into a clean 2 ounce bottle and add 50 c.c. of distilled water.

12. A microscope with a good microscopie light. If reflected light is preferred, a 100 watt, frosted, Mazda electric light bulb, provided with a shade or a small photo flood bulb, is recommended.

#### TECHNIQUE

*Fixing and Embedding.*—Three grams of egg albumen crystals are dissolved in 10 c.c. of tap water. This quantity of emulsion is sufficient for one large-sized block, or two small ones. Complete solution of the crystals takes from forty-five to sixty minutes, unless the mixture is stirred frequently. This time-consuming factor can be obviated by making up reserve quantities of the emulsion which can be preserved at refrigeration temperatures for several weeks.

\*This can be obtained from Benjamin T. Terry, 1901 N. Yakima Ave., Tacoma, Wash.



Containers for blocking are easily made by folding 2½ by 4 inch pieces of cellophane or patapar into the shape of cubical boxes (Fig. 1). The box is placed in a small dish, such as the one-ounce ointment jar. The emulsion of egg protein is poured into the box, and the tissue (fresh or fixed) is placed in the emulsion. A single piece of tissue, as large as one square centimeter or numerous small fragments of tissue, such as small bits of endometrium, can be embedded into a single block. The coagulant, 40 per cent formalin, is poured into the dish around the box. The level of the formalin should be slightly below the level of the emulsoid. By dialysis the formalin gradually permeates the emulsoid, fixing the tissue and coagulating the egg protein to form a solid block. Hardening sufficient for cutting requires from two to eight hours, depending on the temperature of the media. (After a little experience in palpating blocks, the degree of hardness sufficient for cutting is easily determined.)



Fig. 2.—Low-power view of an endometrial polyp.

In an incubator at 140° F. a sufficient hardening can be secured in about two hours, while at a temperature of 70° F. adequate hardening may take from eight to ten hours. The box containing the egg emulsion and tissue may, however, be left in the formalin for twenty-four hours or more without danger of excessive hardening. When sufficiently hardened, the block is held in running water for a few seconds to remove the excess formalin. The cellophane or patapar wrapper peels off easily. After trimming, the block is ready for cutting. When small fragments of tissue, such as strips of endometrium, are embedded, it is usually more convenient to divide the block into two or more squares before sectioning. If it is not convenient to cut sections immediately, the block should be immersed in water to prevent drying and cracking. Any portion of a block before or after cutting can be preserved in tap water for days, or in 5 per cent formalin for months. Tissue embedded by this method is easily re-

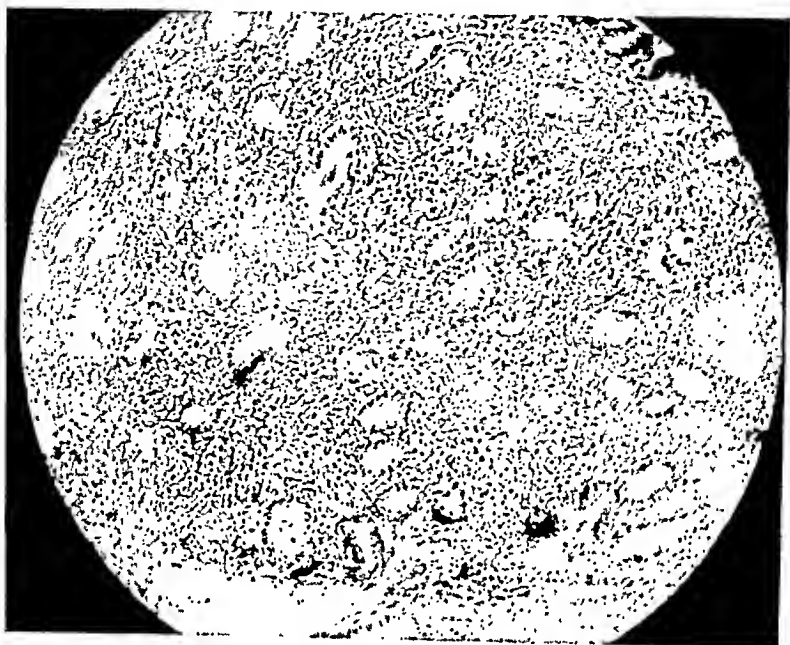


Fig. 3.—Medium-power view of endometrium secured by suction curettage. Chronic endometritis.



Fig. 4.—High-power view of cervical tissue (biopsy). Chronic cervicitis.

moved from the embedding substance and can be reblocked (for the purpose of securing permanent sections) by some other means, such as the slow paraffin method of impregnation.

*Cutting.*—The block can be sectioned easily by freehand cutting with an ordinary, sharp old-fashioned razor. With little experience satisfactory sections, measuring a fraction of a millimeter in thickness, can be obtained. Uniformly thinner and better sections can be obtained, however, by employing Terry's<sup>4</sup> safety razor blade microtome. Wetting the block frequently facilitates cutting. Sections may be washed and stained immediately after cutting or they may be placed in water to prevent drying until convenient to stain.

*Staining.*—Terry's neutralized polychrome methylene blue is a most satisfactory preparation. Only one surface of a section is stained. Overstaining or staining more than one surface hinders proper transillumination and clear visualization of the tissue. To stain, place a section on a thin slab of wet sponge. If not previously washed, gently spurt a few drops of water on the exposed surface. Excessive water will be absorbed by the sponge. The stain may be painted on with a fine-pointed camel's-hair brush or sprayed on with an ordinary atomizer. Adequate staining is secured in from one to three seconds. The intensity and depth of staining are controlled by washing with water. As a rule, properly stained sections are secured by washing almost immediately after applying the stain. Washing is easily accomplished by spurling a medicine dropper or two full of water upon the stain-covered surface. The underlying sponge, if sufficiently wet, will absorb excessive stain and prevent staining of the undersurface of the section. After staining, the section must be mounted and examined immediately, since it fades fairly rapidly.

*Mounting.*—The section is placed on a cover slip with its stained surface next to the glass. The cover slip is then inverted and mounted on an ordinary microconcavity slide with the section in the cavity. It is now ready for transillumination and examination. If difficulty is experienced in transillumination, the section either is too thick or is overstained.

*Examination.*—While this method of preparing sections is most satisfactory for the examination of tissues under low-power magnification, excellent high-power views of most tissues can be obtained if the sections are thin enough and stained properly. Photomicrographs of sections prepared by this technique are generally unsatisfactory because of thickness of sections and the tendency of the stain to fade rapidly. Figs. 2, 3, and 4 are photomicrographs of sections photographed immediately after staining. Obviously, the interpretation of sections prepared by this method requires as much knowledge of histology and pathology as is needed for the correct interpretation of sections prepared by other methods.

#### SUMMARY

1. A technically simple, inexpensive method of preparing tissues for microscopic examination is presented.
2. The method is applicable in the office as well as in the laboratory.
3. Because of the time required for embedding, the method cannot be compared with frozen section technique for speed.

4. With little experience sections can be secured that are as good as, if not better than, the average frozen section.

5. Serial sections of biopsy specimens can be secured quickly and at less expense than by any other method I know.

6. This method should be particularly useful in the office practice of surgeons and gynecologists, who through adequate training, have acquired a good knowledge of micropathology. For instance, in my office it has been particularly useful for the examination of biopsy specimens of lesions of the vulva, vagina, and cervix, as well as for the examination of endometrium obtained by suction curettage.

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I wish to acknowledge my indebtedness to Dr. Benjamin T. Terry for his helpful interest and counsel.

## A STUDY OF TYPE HEMOLYTIC REACTIONS OF HUMAN RED BLOOD CELLS IN DISEASE\*

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OF THE many methods and great variety of reagents that have been used to test the fragility of red blood cells only one has been found to have practical value as an aid in diagnosis. Hypotonic solutions of sodium chloride are used to test the fragility of erythrocytes in hemolytic jaundice and obstructive jaundice and have been reported as a prognostic guide in pneumonia.<sup>1</sup> This test has been applied to many diseases, but a review of literature does not reveal any systematic study in which several types of hemolytic agents were used to test the strength of red blood cells from a number of different diseases. This study is based upon the use of a comparatively short method which is adaptable for the use of several kinds of hemolytic agents. The method has been applied to red blood cells from normal individuals and patients with various diseases.

#### REAGENTS

As a neutral salt sodium chloride was used in hypotonic solutions. Its hemolytic property is believed to be due to osmosis, although various explana-

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tions are presented concerning its mode of action.<sup>2,3</sup> Because it has been so widely used by other investigators, it is a good "yardstick" for comparison of results.

The hemolytic properties of acids and bases are believed to be due to a more complex action. It may be that they change the physical state of the cell proteins<sup>4</sup> or that the action may be concerned with the respective H and OH ions of the acids and bases.<sup>5</sup> Lactic acid and sodium carbonate were chosen to represent the acid and base hemolysins, respectively, because they are less toxic than the stronger agents of this group. A reaction which took place between lactic acid and the hemolysis products clouded the system and made accurate readings difficult. Because of this, it was excluded from this report.

Sodium oleate was used as a representative of the soaps whose lytic properties are now attributed to their action on proteins.<sup>4</sup> This is contrary to the former interpretation, which considered the effects of those active hemolysins to be derived from their lipid solvent characteristics.

Saponin represented the glucoside lysins. Its hemolytic properties are probably due to its reaction with the cell proteins to a far greater extent than with the lipoids.

Alcohol was used as a lipid solvent hemolysin. It is a rather weak hemolysin, but gives a very definite end point. A protein precipitate forms in the system a short time after hemolysis is complete, but does not enter into consideration within the time limits of the method to be described.

#### METHOD

From 3 to 5 c.c. of blood were drawn from the anteeubital vein into dry, sterile syringes and transferred immediately to sterile test tubes containing potassium oxalate. It was found that this anticoagulant does not alter the lytic properties of red blood cells. The cells of the fresh, oxalated blood were washed four times in 0.9 per cent sodium chloride solution and centrifuged ten minutes after each washing at a medium speed. At the rate used (1,400 to 1,500 r.p.m.) there is very little mechanical damage to the cells. An average of 2.4 c.c. of packed cells was recovered from 5 c.c. of normal blood. Suspensions of washed cells were prepared in the proportion of 2.4 c.c. of erythrocytes to 97.6 c.c. of 0.9 per cent sodium chloride solution.

TABLE I

| AGENT                              | PER CENT CONCENTRATION |       |       |       |       |       |       |       |       |       |
|------------------------------------|------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|                                    | 1                      | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    |
| Test tube No.                      | 0.075                  | 0.150 | 0.225 | 0.300 | 0.375 | 0.450 | 0.525 | 0.600 | 0.675 | 0.750 |
| Sodium chloride                    | 0.833                  | 0.667 | 0.500 | 0.433 | 0.333 | 0.287 | 0.250 | 0.220 | 0.200 | 0.180 |
| Saponin ( $\times 10^{-2}$ )       | 0.225                  | 0.180 | 0.135 | 0.090 | 0.067 | 0.037 | 0.030 | 0.022 | 0.015 | 0.007 |
| Sodium carbonate                   | 0.125                  | 0.100 | 0.083 | 0.067 | 0.050 | 0.042 | 0.033 | 0.025 | 0.017 | 0.008 |
| Sodium oleate ( $\times 10^{-2}$ ) | 28.50                  | 23.75 | 20.58 | 15.83 | 12.67 | 7.92  | 6.33  | 4.75  | 3.16  | 1.58  |
| Ethyl alcohol                      |                        |       |       |       |       |       |       |       |       |       |

A series of ten hemolysis tubes was set up for each lysin, consecutively, using varied quantities of stock solutions with 0.9 per cent sodium chloride as diluent. These tubes, together with a tube of red blood cell suspension, were placed in a constant temperature bath set at  $37^{\circ}\text{C.} \pm 0.2$  degree. When the contents of the tubes reached a temperature of  $37^{\circ}\text{C.}$ , an equal quantity of

the suspension was added to each tube of lysin and mixed by inverting once. All measurements were made with dry, chemically clean, burettes and pipettes. Care was used to keep all conditions as uniform as possible.

Table I shows the concentrations of each lysin.

The insulated water bath was arranged so that a strip of glass was left free across the front and back, with an equal distribution of light passing through the container. If hemolysis was completed in less than thirty minutes, the time necessary for completion was recorded. It is relatively simple to read complete hemolysis because the contents of the tube become clear. At the end of a half-hour period the degree of hemolysis in all tubes was estimated and recorded. The thirty-minute period proved optimum for securing hemolysis in a fairly wide dilution range of the lysins, and allowed less time for a new colloid-crystalloid adjustment in the cells, or for change of any hemolysis products set free in the tubes.

### RESULTS

Average values for each lysin were first made by testing their effect on erythrocytes of normal individuals. Thirty-five persons were used, ranging in age from 5 to 84 years. The level of hemolysis of different individuals varied considerably, but variation in age had no significant effect on the reaction of the cells.

TABLE II  
COMPLETE HEMOLYSIS

|                        | NO. | SAPONIN | SODIUM CARBONATE | SODIUM OLEATE | ETHYL ALCOHOL | SODIUM CHLORIDE |
|------------------------|-----|---------|------------------|---------------|---------------|-----------------|
| Normal                 | 35  | 0.342   | 0.066            | 0.055         |               |                 |
| Pernicious anemia      | 9   | 0.344   | 0.074            | 0.055         | 15.24         | 0.219           |
| Secondary anemia       | 8   | 0.327   | 0.078            | 0.062         | 15.48         | 0.175 +         |
| Obstructive jaundice   | 4   | 0.310   | 0.084            | 0.064         | 15.01         | 0.206           |
| Pneumonia              | 10  | 0.285   | 0.064            | 0.052         | 15.83         | 0.113 +         |
| Scarlet fever          | 10  | 0.341   | 0.072            | 0.062         | 15.83         | 0.195           |
| Sepsis                 | 9   | 0.372   | 0.073            | 0.064         | 14.88         | 0.165 +         |
| Meningitis             | 10  | 0.314   | 0.055            | 0.057         | 16.77         | 0.175 +         |
| Tuberculosis           | 10  | 0.373   | 0.072            | 0.054         | 14.25         | 0.188           |
| Carcinoma              | 10  | 0.383   | 0.076            | 0.063         | 15.83         | 0.203           |
| Sarcoma                | 9   | 0.355   | 0.082            | 0.055         | 15.83         | 0.188           |
| Syphilis               | 9   | 0.319   | 0.071            | 0.053         | 16.01         | 0.175 +         |
|                        |     |         |                  |               | 15.48         | 0.225           |
| THRESHOLD OF HEMOLYSIS |     |         |                  |               |               |                 |
| Normal                 | 35  | 0.195   | 0.020            | 0.036         |               |                 |
| Pernicious anemia      | 9   | 0.193   | 0.015            | 0.026         | 10.04         | 0.456           |
| Secondary anemia       | 8   | 0.193   | 0.024            | 0.033         | 7.91          | 0.525           |
| Obstructive jaundice   | 4   | 0.180   | 0.024            | 0.035         | 9.10          | 0.450           |
| Pneumonia              | 10  | 0.197   | 0.031            | 0.035         | 10.29         | 0.375 +         |
| Scarlet fever          | 10  | 0.194   | 0.014            | 0.038         | 11.55         | 0.353 +         |
| Sepsis                 | 9   | 0.207   | 0.017            | 0.038         | 8.23          | 0.420           |
| Meningitis             | 10  | 0.180   | 0.009            | 0.034         | 8.79          | 0.450           |
| Tuberculosis           | 10  | 0.180   | 0.012            | 0.030         | 9.49          | 0.435           |
| Carcinoma              | 10  | 0.204   | 0.023            | 0.037         | 10.92         | 0.398           |
| Sarcoma                | 9   | 0.193   | 0.024            | 0.031         | 8.86          | 0.450           |
| Syphilis               | 9   | 0.191   | 0.029            | 0.038         | 9.49          | 0.458           |
|                        |     |         |                  |               | 9.85          | 0.400           |

± indicates statistical significance.

Tests were then made of the red blood cells of patients with various diseases. Table II gives a summary of the average values for beginning and completion of hemolysis.

## DISCUSSION

In analyzing Table II two conditions are evident which lower the statistical significance of the results. One is the limited number of cases studied, only 35 for the normal figures, and still less for results in each of the diseases studied. It is realized that increasing the number of cases taken at random improves the statistical accuracy. Practically, however, one is limited as to the number of patients available. Furthermore, the objection as to numbers of cases is mitigated by the rather low swings from average in the individual case studies.

A further statistical objection relates to the lack of fineness of gradation in the hemolysis tests themselves when only ten tubes are used. However, the tests were formulated so that the hemolyzing zone would be found in the central tubes in normal cases. A swing to the right or left in abnormal cases would still be recorded within the given number of tubes. In a very large series of tests, adding still further to the number of tubes obviously would improve the statistical evidence of trends in the results within narrow limits, but would not improve the accuracy of the test.

It appears from the recorded results that the red blood cells are rather well adjusted to possible changes in the blood during disease. At least there is little evidence of variation from normal in the resistance of the cell when exposed to substances which affect either osmotic factors or protein or lipid constituents of the cells. Even when the averages were altered significantly, the range of concentration at which hemolysis occurred was, in most instances, within the range at which cells from normal persons were hemolyzed.

This seems to be the case in reports of other investigators also; that is, few have found any consistent reaction of the red blood cells that would be of diagnostic value. Trends are indicated but individual cases often vary from increased to decreased resistance when testing several cases with the same disease, as reported by Hill<sup>6</sup> and Simmel<sup>7</sup> on pernicious anemia. Randall<sup>8</sup> found the red blood cell resistance in pernicious anemia to be decreased, while Giffin and Sanford,<sup>9</sup> and Cassells<sup>10</sup> report an increased resistance. On the other hand, Daland and Worthley<sup>11</sup> record normal maximum and decreased minimum resistance. All these investigators used hypotonic solutions of sodium chloride to test fragility. When saponin was the hemolysin, the reaction either was normal or showed a decrease in resistance.<sup>12-14</sup>

Needles<sup>1</sup> found a definite relationship in pneumonia between the resistance of red blood cells to hypotonic sodium chloride and the amount of lung tissue involved. Although there is a significant increase in mean resistance indicated in Table II, the data in these experiments show that many cases fall within normal range, and there is no such correlation of the resistance and extent of lung involvement.

Different results might have been obtained had the cells been unwashed, in which case possible changes in the serum would have played an important part. However, since this group of experiments was on the cells themselves, the serum had to be eliminated by washing in order to make the conditions of the experiment as uniform as possible.

## SUMMARY

A method for testing fragility of red blood cells with a variety of type hemolytic agents is presented.

The hemolytic level of human red blood cells with various lysins is indifferent to the factor of age, from 5 to 84 years.

Results of applying this method to red blood cells from patients with pernicious anemia, secondary anemia, obstructive jaundice, pneumonia, scarlet fever, meningitis, sepsis, tuberculosis, carcinoma, sarcoma, and syphilis are recorded.

No significant changes of new diagnostic value were found in the erythrocytes with the method employed.

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## SIMPLIFIED METHOD FOR BIOLOGICAL DIAGNOSIS OF PREGNANCY\*

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THE biologic diagnosis of pregnancy, which I discovered in 1927, is based on the presence of gonadotropin in the urine and in the serum of pregnant women. My original method is as follows:

For the examination of every urine specimen, one needs five immature female mice, 3 to 4 weeks old and weighing 6 to 8 Gm. The total amounts injected subcutaneously into the five animals vary, but in every case six equal injections are made, distributed over forty-eight hours.

The first animal receives six times 0.2 c.c. (total 1.2 c.c.), the second animal six times 0.25 c.c. (total 1.5 c.c.), the third and fourth animals six times 0.3 c.c. (total 1.8 c.c.), and the fifth animal six times 0.4 c.c. (total 2.4 c.c.). Vaginal smears are made from the third day on. After ninety-six hours the animals are autopsied.

The ovaries are examined with the naked eye, or better, with a hand lens. The presence of hemorrhagic follicles and of corpora lutea is considered indicative of pregnancy. This method gives from 98 to 99 per cent correct results, and only from 1 to 2 per cent erroneous results.

Application of the same method to female immature rats does not give better results than with the mouse. All modifications of the method are based on the same principle, namely, looking for gonadotropin in the urine or the blood.

The modification of Friedman, which uses the adult female rabbit, can give a positive result twenty-four hours after intravenous injection of from 7 to 10 c.c. of urine. The modification of this Friedman method, as used by Brouha, gives the result in forty-eight hours. One injects 5 c.c. of urine on the first day, and 5 c.c. on the second day, into the ear vein of the female rabbit. Ovulation and the presence of hemorrhagic follicles are signs of the action of the gonadotropin.

Other modifications are the ones by Brouha-Simonnet<sup>1, 2a</sup> and Kraus.<sup>2</sup> These authors use immature male mice or rats whose seminal vesicles increase in volume after daily injections of pregnancy urine during seven or eight days. This increase in the volume of the seminal vesicles is caused by the action of gonadotropin on the testicles, especially on the interstitial cells of Leydig, which manufacture the male hormone; this male hormone in turn produces an overgrowth of the seminal vesicles.

Bourg<sup>3</sup> has employed a male rat and a female rat, both immature. He injects 1.1 c.c. of urine daily for five days and studies the seminal vesicles in the male and the ovaries in the female.

\*Translated from the French by Alfred Plaut, Beth Israel Hospital, New York.  
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Shapiro and Zwarenstein<sup>4</sup> have employed the South African clawed toad, *Xenopus laevis*, as a test animal. After injection of pregnancy urine, the animal will lay eggs after ovulation has been stimulated. The result can be studied six to eight hours after the injection. Elkan<sup>5</sup> has obtained satisfactory results with this method.

If I have tried to find a simplification of the method, my reason for this was not the hope of improving the results. External factors have induced me to look for a more economical method as far as material, working time, and expenses are concerned, in short, a method adapted to present conditions. The reasons are the following:

My original method with the five mice, and its modifications, require much work because injections have to be made frequently, and the same applies to the exploratory laparotomy in the female rabbit.

TABLE I

|  | NO.<br>OF<br>CASES | RAT TEST   | RABBIT TEST                              | REMARKS  |
|--|--------------------|--|--|--|
| Normal pregnancy   |                    |  |  |  |
| 6-8 weeks  | 13                 | Of these 35 tests,<br>33 were positive,<br>none negative.<br>Two rats died | 24 positive                              | Two false negatives<br>with the rabbit test  |
| 9-12 weeks   | 11                 |  | 2 negative                               |  |
| 13-20 weeks  | 6                  |  | 2 rabbits died                           |  |
| 28-36 weeks  | 4                  |  | In 7 cases no rabbit test was made       |  |
| Unknown  | 1                  |  |  |  |
| Abnormal pregnancy<br>3 months tubal, one<br>day after operation<br>for rupture  | 1                  | Positive   | Not made                                 |  |
| Two months tubal<br>gestation  | 1                  | Positive   | Positive                                 |  |
| Same case—<br>5 weeks later  | 1                  | Negative   | Negative                                 | Operation revealed<br>an old tubal abortion  |
| Missed abortion<br>(intrauterine)  | 1                  | Negative   | Negative                                 |  |
| Nonpregnant women<br>(amenorrhea, menorrhagia,<br>menopause, castration, myomas) | 30                 | Negative   | 10 not made<br>17 negative<br>3 positive | Three false negatives<br>with the rabbit test<br>In the menopause and<br>after castration the<br>rat test was negative<br>even with 1 c.c. of<br>urine |
| Teratoma of testicle   | 1                  | Negative   | Negative                                 |  |

NOTE: There were no false results with the new rat test (two rats died).

Buying the animals is expensive. That is why I have put to myself the question how it is possible to simplify the method by using immature female rats four to five weeks old.

Applying the original method to immature rats has never appeared advantageous to me. The hemorrhagic follicles are often absent, and sometimes the corpora lutea are difficult to distinguish from the interstitial tissue with the naked eye.

Why then did I choose the female rat as a test animal?

The Third International Conference on the Standardization of Hormones of the Health Organization of the League of Nations,<sup>6</sup> at which I was present, recommended creating an international standard for the gonadotropic sub-

stance obtained from pregnancy urine. The Conference decided that the international unit would be defined as representing the specific gonadotropic activity of 0.1 mg. of a standard prepared by mixing numerous samples. This standard has been examined by numerous workers in order to determine its content of rat units.

It is agreed that a rat unit is the number of international units which produces in 50 per cent of the immature rats, injected subcutaneously during two or three days, vaginal keratinization. Formation of keratinized cells in vaginal smears is known as the Allen-Doisy test. It must be understood, however, that preparations of gonadotropin from pregnancy urine must not contain estrogen. The absence of estrogen can be proved by injecting the gonadotropin into castrated animals. Under these conditions the method for determination of gonadotropin in pregnancy urine can be used for commercial preparations in spite of the fact that the method is based on an indirect effect and not on an ovarian change. In the method I have called the simplified method of the pregnancy reaction, I rely entirely upon vaginal smears (the Allen-Doisy test) without entering into the question whether the reaction is caused by gonadotropin in the urine or by estrogen. As I have shown, the estrogen also is present in considerable quantity in the pregnancy urine. It also is possible that both hormones act simultaneously.

The method is the following: To two female immature rats, 4 to 5 weeks old, weighing 22 to 35 Gm. (the weight figures apply only to our strain), one subcutaneous injection of 0.5 c.c. of the urine to be examined is given. Vaginal smears of both rats are made seventy-two, eighty-four, and ninety-six hours after this single injection.

If the urine comes from a pregnant woman, only epithelial cells without free mucus and without leucocytes in the vaginal smear are found after seventy-two hours (often already after sixty-six hours). After eighty-four to ninety-six hours keratinized cells (seldom mixed with a few epithelial cells) are found. The keratinized cells constitute the criterion of the positive reaction.

The animals need not be killed. They may be used for other experiments, for instance castration.

If an autopsy is performed, easily visible follicles in the ovaries are found. Corpora lutea are seldom seen, and the uterus is found enlarged. Since animals of the same age and weight, which have not been injected, may also show visible follicles, this fact is not of diagnostic value. Only the fact that the vaginal smears contain the keratinized cells after a single injection of 0.5 c.c. of urine is important and can be used for the diagnosis of pregnancy.

The following objections may be raised against this method:

1. The Allen-Doisy test proves only an estrogenic effect. In the castrated animal (rat, mouse) it proves that the fluid injected contains an estrogenic substance.

In the noncastrated immature animal the Allen test indicates that in the injected fluid there is either an estrogenic substance acting directly on the vagina or a gonadotropic follicle-stimulating substance which acts by the intermediation of the ovary on the vagina. Or, finally, a hypophyseotropic substance which

stimulates the anterior lobe of the pituitary and makes it produce and secrete follicle-stimulating hormone. This substance thus would act upon the vagina by way of two intermediary organs, namely, the pituitary and the ovary.

Estrogenic substance is not eliminated in the urine in sufficient quantity, so that 0.5 c.c. in a single subcutaneous injection could lead to a positive Allen-Doisy test except in pregnancy. Normal women excrete less than 1,000 units. The presence of from 300 to 500 mouse units per liter indicates abnormally increased secretion of estrogen.

The amount of estrogen also is increased in women who bear granulosa cell tumors of the ovary. Several hundred mouse units have been found per liter of urine, but so far one has not observed an excretion of 6,000 units per liter, and this is the minimum necessary for giving a positive reaction in the immature rat after a single injection of 0.5 c.c. The abnormal estrogen secretion which sometimes is found in women who have follicular cysts, or fibrocystic ovaries, remains below 6,000 mouse units per liter.

Only in the second half of pregnancy does the elimination of estrogenic substance rise to several thousand mouse units (6,000 to 40,000, and higher) per liter of urine.

Thus, if an immature rat, after receiving an injection of 0.5 c.c. of urine of a woman, gives a positive Allen-Doisy test, one can conclude that it was the urine of a pregnant woman that has produced this reaction.

2. One can object that the follicle-stimulating gonadotropic substance of hypophyseal origin, which also is eliminated with the urine, might falsify results. But this elimination usually does not exceed 30 rat units per liter; it seldom reaches 75 rat units in the normal woman. In women with physiologic or artificial menopause (operation or x-ray) the amount of follicle-stimulating hormone is elevated in the urine. But so far no elevation to 2,000 rat units, which would correspond to 4,000 mouse units, has been found. The highest known figures are 1,000 mouse units. One rat unit of the follicle-stimulating hormone corresponds to two or three mouse units; conversely, in the case of gonadotropin obtained from pregnancy urine (placental origin), one mouse unit corresponds to five rat units.

Occasionally women with primary amenorrhea eliminate gonadotropic follicle-stimulating substance in a large amount, but in my experience not more than 1,000 mouse units. Furthermore, clinically in these women who always have an atrophic uterus, the question of pregnancy does not present itself.

3. Increased elimination of follicle-stimulating hormone (and seldom also of luteinizing hormone) in women with cancer of the cervix or cancer of the vulva has been observed. I have examined about 100 such women, most of whom were in menopause. I have seldom found increased elimination in cancer patients who still menstruated. The figures in these cases did not exceed 1,000 mouse units. I have not yet done quantitative studies of these cases with immature rats, but I do not believe that we could obtain a wrong result with this simplified method by injecting only 0.5 c.c. of urine.

4. The cases of persisting corpus luteum sometimes have given a positive reaction with mice or rabbits. These cases are very rare and one has to wait

a long time before one encounters a case in which the differential diagnosis between extranterine pregnancy and persistent corpus luteum is of importance. In such cases the simplified method perhaps will be better than the other methods.

5. Cases of chorio-epithelioma and of teratomas of ovaries, and of other organs which give a positive reaction in the mouse and the rabbit, have not yet been examined with the rat method. In tumors which contain chorionic cells no biologic method can give a differential diagnosis between pregnancy and tumor.

6. The combined excretion of estrogen and gonadotropin in quantities, which would give a positive reaction, in our present stage of knowledge cannot be expected, except for chorio-epithelioma or teratoma.

The physician must know that such rare cases exist and must consider them during his clinical examination.

The biologic pregnancy test must not replace exact clinical examination. Close collaboration between the physician and the laboratory man is always necessary.

Thus, we have seen that all the objections against the simplified method can be refuted.

I have chosen 0.5 c.e. for injection. In some cases I have injected only 0.2 c.e., and I have obtained positive results, except in one case in which 0.5 c.e. gave a positive result while with 0.2 c.e. the result was negative. I must continue these tests for if a lower dose than 0.5 c.e. should give the same results, all objections against the method would become even weaker. Meanwhile, I propose the dosage of 0.5 c.e.

In five cases of pregnancy I have given a single injection of 1 or 2 c.e. of urine to male immature rats in order to see whether or not the simplified method is also applicable to the male. This method would have to be based on the increase in size of the seminal vesicles (Brouha-Simonnet, Kraus). Since I could not see such an increase ninety-six hours after the injection, I believe that the simplified method cannot be applied to the male immature rat.

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# AN IMPROVED POLYCHROME METHYLENE BLUE EOSINATE\*

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CONSIDERABLE variation in the effectiveness of staining malaria parasites is found in using different lots of Wright's stain purchased on the open market. Frequently malaria parasites stained by Wright's technique are either understained or are otherwise poorly differentiated. Experiments reported here were undertaken in an attempt to produce a stain with a strong affinity for blood parasites. Several eosinates of methylene blue polychromed with various agents were made and tested. The eosinate of methylene blue polychromed with sodium hydroxide was found to be unstable and of inferior staining quality. However, methylene blue polychromed with sodium hydroxide and then treated with sodium bromide, prior to the addition of the eosin solution, proved effective. Six lots prepared by this procedure have given the desired staining effect, and after more than six months each lot is still stable both in powder and in solution. It is believed, therefore, that this technique provides a reliable method for uniform staining of blood parasites.

The stain is prepared as follows: One gram of methylene blue chloride (certified) is dissolved in 100 c.c. of N/100 sodium hydroxide (carbonate free) in a ground glass-stoppered bottle of about 500 c.c. capacity. Best results are obtained when not more than one-quarter of the bottle is filled by the solution during the polychroming process. The solution is heated in a water bath at 55° C. for two and one-half hours. The bottle stopper is loosened slightly before heating and at half-hour intervals the solution is shaken vigorously for about a minute. At the end of two and one-half hours, 1 Gm. of sodium bromide is added and dissolved in the polychromed methylene blue, and the solution is again heated in the water bath for another two and one-half hours, allowed to cool, and filtered.

To the polychromed methylene blue filtrate is added 60 c.c. of a 1 per cent aqueous solution of eosin "Y" (certified water and alcohol soluble), and mixed well with a glass stirring rod. More eosin is added in 5 c.c. amounts until the solution between the precipitated particles has a reddish tinge. This is best observed by letting a little of the precipitate from the solution run down the inside of the upper portion of the beaker. Generally, about 80 c.c. of the eosin solution give the desired maximum precipitation of the eosinate. The precipitate is allowed to stand for from eighteen to twenty-four hours. The precipitate is then collected on hard filter paper in a Buchner funnel with vacuum until the precipitate is sufficiently dry to crack. The precipitate is allowed to dry completely in the incubator at 37° C., and then ground to a fine powder in a mortar. The powder is stored in a small tightly stoppered glass bottle.

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In making the stain 0.1 Gm. of the powder is dissolved in 60 c.c. of absolute methyl alcohol, acetone free. The stain is allowed to stand two to three days, occasionally shaken, until the dye appears to be completely dissolved. The stain solution is filtered and stored in a small tightly stoppered bottle.

#### STAINING TECHNIQUE

1. Cover a thin blood film with 0.5 c.c. of stain and allow to fix for one minute.

2. Dilute the stain with 2 c.c. of phosphate buffer solution, mix thoroughly, and allow to stain for three minutes. A pH range of 6.4 to 7.0 for the phosphate buffer diluent is satisfactory. When suitable diluent is added, an iridescent scum is formed over the stain. Best staining results are obtained with freshly made diluents, not more than one week old.

3. Wash by flooding with distilled water. Air dry and examine.

This technique is simple and rapid, and has yielded uniform and superior results. It is easy to obtain stained preparations free from precipitated stain.

## CHEMICAL

### A COMPARISON OF THE BODANSKY AND FISKE-SUBBAROW METHODS FOR THE DETERMINATION OF INORGANIC PHOSPHORUS\*

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DURING the course of our investigations of the physiologic functions in schizophrenic patients, a study was made of the serum phosphatase levels. This necessitated a change in the technique of phosphorus determinations from the Fiske-Subbarow<sup>1</sup> method previously used to that of Bodansky.<sup>2, 3</sup> As the phosphorus values seemed to run lower by the new method, duplicate analyses of 19 sera were made by the two methods. The results of these analyses are given in Table I. The values given under the stannous chloride method have been corrected in accordance with the instructions of Bodansky. Because two cases did not have duplicates by both methods, the statistical analysis was carried out for only 17 samples.

It will be noted that despite these corrections the use of the Bodansky technique gives lower values than that of Fiske and Subbarow. The correlation between the two methods is 0.87, and the values of the Fiske method can be calculated from those obtained by the Bodansky technique after applying his corrections by applying the following regression equation:

$$Y = 0.7784 X + 0.9511$$

where Y = inorganic phosphorus Fiske, and

X = inorganic phosphorus Bodansky corrected.

The equation for the uncorrected Bodansky value is

$$Y = 0.973 X' + 0.327,$$

where X' is the uncorrected Bodansky value. The error of estimation by these equations is  $\pm 0.18$  mg.

The mean value obtained by the Bodansky method was  $3.12 \pm 0.1$  mg. per cent, while that by the Fiske method was  $3.31 \pm 0.1$ . These values were from a group of chronic schizophrenic patients and are lower than those found in the acute stage of the disease.<sup>4</sup>

Since the two series show a high correlation, in order to test for significance of the difference between the means, it is necessary to use the expanded formula for the standard error of the difference:

$$\sqrt{E_1^2 + E_2^2 - 2r(E_1E_2)}$$

where  $E_1$  and  $E_2$  are the standard errors of the means, and  $r$  is the correlation coefficient between the two sets of observations. In this case the standard error

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of the difference is 0.051, and since the difference between the means is 0.19, the ratio between these is 3.73, which is highly significant. The probability that this difference could occur by chance is less than 0.0003.

The duplicate determinations by the Fiske method using 1, 2, 4 aminonaphthosulfonic acid as the reducing agent show much closer agreement than do those using stannous chloride. The variance within samples for the first method was 0.0045, while that for the second method was 0.0138. This difference in variance of three times is highly significant in a sample of 17 duplicates, and indicates that the precision of the Fiske method is much higher than that of the Bodansky method.

TABLE I

|        | SERUM INORGANIC PHOSPHORUS<br>(mg. per 100 ml.) |        |              |        |
|--------|---|--------|--------------|--------|
|        | BODANSKY METHOD                                 |        | FISKE METHOD |        |
| H. A.  | 3.05  | (3.05) | 3.32         |        |
|        | ----  |        | 3.26         | (3.29) |
| E. B.  | 3.49  | (3.52) | 3.78         |        |
|        | 3.55  |        | 3.96         | (3.87) |
| A. B.  | 3.57  |        | 3.96         |        |
|        | 3.72  | (3.65) | 3.96         | (3.96) |
| S. C.  | 3.19  |        | 3.48         |        |
|        | 3.34  | (3.27) |              | (3.48) |
| W. D.  | 3.99  |        | ----         |        |
|        | 4.07  | (4.03) | 4.06         |        |
| A. Du. | 2.86  |        | 4.08         | (4.07) |
|        | 3.00  | (2.93) | 2.84         |        |
| A. O.  | 3.32  |        | 2.84         | (2.84) |
|        | 3.28  | (3.30) | 3.16         |        |
| T. G.  | 3.30  |        | 3.18         | (3.17) |
|        | 3.36  | (3.33) | 3.60         |        |
| G. H.  | 3.19  |        | 3.60         | (3.60) |
|        | 3.26  | (3.23) | 3.46         |        |
| A. Da. | 2.90  |        | 3.46         | (3.46) |
|        | 3.19  | (3.05) | 3.06         |        |
| H. H.  | 2.81  |        | 3.06         | (3.06) |
|        | 2.69  | (2.75) | 2.78         |        |
| H. K.  | 2.90  |        | 2.78         | (2.78) |
|        | 2.84  | (2.87) | 2.66         |        |
| S. L.  | 3.67  |        | 2.66         | (2.66) |
|        | 3.47  | (3.57) | 3.60         |        |
| T. M.  | 3.00  |        | 3.56         | (3.58) |
|        | 3.05  | (3.03) | 3.04         |        |
| F. P.  | 3.17  |        | 3.04         | (3.04) |
|        | 3.19  | (3.18) | 3.40         |        |
| R. A.  | 3.00  |        | 3.28         | (3.34) |
|        | 2.90  | (2.95) | 3.48         |        |
| W. B.  | 2.54  |        | 3.60         | (3.54) |
|        | 2.04  | (2.29) | 2.74         |        |
| P. B.  | 2.67  |        | 2.76         | (2.75) |
|        | 2.71  | (2.69) | 3.44         |        |
| M. C.  | 2.75  |        | 3.14         | (3.29) |
|        | 2.77  | (2.76) | 3.30         |        |
|        |   |        | 3.30         | (3.30) |

The reduction by stannous chloride was followed in the photoelectric colorimeter. The maximum reduction occurred almost instantaneously and remained constant for approximately ten minutes. Slight variations in the strength of acid or molybdate produce marked changes in the depth of color. These changes are not so important when the unknown is read against a standard solution but can lead to considerable errors when read in a photoelectric colorimeter and

compared to a standard curve. The method, however, is about six times more sensitive than the Fiske method, so that determinations can be made on smaller amounts of material.

The deviation from Beer's law is due to the reduction of the uncombined molybdic acid, and the precautions outlined by Bodansky must be followed in order to minimize this reduction, as shown in the blank. All alkaline solutions must be stored in paraffined bottles and the N 10 sulfuric acid should be kept in a pyrex glass container to prevent the contamination of the solutions by silicates. This effect of silicates has been noted by Roe and Whitmore.<sup>2</sup>

Berenblum and Chain<sup>6, 7</sup> have noted similar difficulties in the use of stannous chloride as a reducer and have devised a method for overcoming this by extracting the combined phosphomolybdic acid with isobutyl alcohol to remove the excess of molybdic acid. This method, though capable of giving accurate results on dilute solutions, requires several extractions and washings, which are somewhat of a nuisance when many determinations must be carried out.

#### SUMMARY

Duplicate analyses for inorganic phosphorus were made on sera from 17 schizophrenic patients by both the Fiske-Subbarow and the Bodansky methods. Readings were made on a Lange photoelectric colorimeter. The deviation from Beer's law produced in the Bodansky method, using stannous chloride as the reducing agent, is not completely compensated for by the corrections applied by Bodansky. The variance of the Bodansky method is 0.0138 as compared with 0.0045 for the Fiske-Subbarow method.

By the use of a photoelectric colorimeter and comparison with a standard curve, the error of the Bodansky method due to deviations from Beer's law may be kept at a minimum provided that extreme care is taken in duplicating the reagents used. A known phosphorus standard should be included in each series of unknowns as a check on the reagents. If a photoelectric colorimeter is not available, reasonably accurate estimations can be made by application of one of the formulas given to the values obtained with a Duboseq type colorimeter.

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## ESTIMATION OF VITAMIN D IN BLOOD SERUM\*

### VITAMIN D IN HUMAN SERUM DURING AND AFTER PERIODS OF INGESTION OF LARGE DOSES OF VITAMIN D

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IN THE two preceding papers of this series<sup>1, 2</sup> a method for the estimation of the vitamin D content of blood serum was described, and data were presented on the vitamin D content of blood sera from persons taking a normal diet without added vitamin D. In 155 samples of such human blood serum the average concentration of vitamin D was found to be 110 U.S.P. units per 100 c.c.; but values between 66 and 165 units per 100 c.c. were regarded as within normal limits, especially when assays of individual samples of serum were based on a small number of line tests. No significant difference was found between the vitamin D content of sera of normal children at ages beyond infancy and the sera of adults.

The present report deals with similar assays of the concentration of vitamin D in the blood serum of adult patients examined at varying intervals during and after periods in which large daily doses of vitamin D were prescribed. These persons were suffering with mild or moderately severe chronic proliferative arthritis of several years' duration. For the opportunity to study them we are indebted to Dr. Stanley E. Dorst, of the Department of Medicine of the University of Cincinnati. While these patients were treated with vitamin D, it was thought that the assay of the vitamin D content of their blood sera at different times might add information concerning the metabolism of vitamin D in the body. The use of large doses of vitamin D in the treatment of chronic arthritis has been described by numerous authors (see review of this subject by Abrams and Bauer<sup>3</sup>). It is not our purpose to discuss here the merits or demerits of this form of therapy, but merely to describe the changes in concentration of vitamin D which were found in the blood serum of these patients following the ingestion of large doses of vitamin D. Assays of vitamin D in single samples of blood serum from children receiving equally large daily doses of vitamin D for various therapeutic reasons have yielded data essentially similar to those reported here.

For use in these investigations two preparations were supplied by the manufacturers. The Winthrop Chemical Company, New York, furnished a solution

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of crystalline vitamin D<sub>2</sub> in oil, 1,000,000 units per gram, and the W. S. Merrell Company, Cincinnati, furnished capsules each containing 50,000 units of vitamin in oil.

### METHODS

In biological assays of blood sera, which may vary considerably in vitamin D content, it is useful to perform preliminary tests to ascertain the range in which the vitamin D concentration falls. Another assay is then performed by administering to a series of rats different amounts of serum, varying by smaller increments within the range of dosage established by the preliminary test. If the results of duplicate line tests in this series do not prove uniform, a third assay may be necessary. Since several weeks may elapse sometimes before the final results are obtained, it is important to prevent bacterial contamination of the serum and also to prevent deterioration of its vitamin D content. Both objectives have been achieved by freezing and drying the serum, either the whole sample or the portion not used in the preliminary assay. Such dried serum samples can be stored and then dissolved at any convenient time when needed for further assays. The drying of the serum offers another advantage if the vitamin D content of the serum is low. In such cases the amounts of serum required to initiate healing in rachitic rats may be so large that its administration to the rats may be difficult. Since the dried material can be redissolved easily in one-fifth its original volume of water, the volume of the serum to be administered can be greatly reduced. This smaller volume also makes possible the administration of the serum directly to the rats in doses accurately measured by pipette, a method of administration preferable to mixing a test material with the rachitogenic diet.

### METHOD AND PROCEDURE FOR DRYING SERUM

A method of drying of biological materials in the frozen state has been described by Shackell<sup>4</sup> as early as 1909. The desiccation of sera and other biological products, including microorganisms, in the frozen state was accomplished by Elser, Thomas, and Steffen.<sup>5</sup> The method was further developed by Flosdorf and Mudd.<sup>6</sup> For our purpose a simple and inexpensive method was desirable, and we used an apparatus and a procedure which are essentially identical with that described by Cooper and Grabill,<sup>7</sup> the only difference being that vials of serum are substituted for the bacterial cultures. In this method the serum is first frozen in a dry ice bath and then dried *in vacuo*, the water being absorbed by a suitable chemical desiccant. The availability of the apparatus, its simplicity of construction and operation, and the economy in time, together with the satisfactory results obtained, prompted us to employ it.

The equipment for the drying of the serum consists of a vacuum pump, a Wouff Bottle of 2 liter capacity, serum vials with stoppers\* (Fig. 1), a desiccant such as "Drierite," glass connections, and other accessories, such as rubber stoppers and pressure tubing. Our routine procedure is as follows:

Five cubic centimeters of human serum are frozen in 10 c.c. vials in a position illustrated in Fig. 1A. In order to insure the proper drying conditions,

\*The serum vial stoppers were purchased from the F. J. Stokes Machine Co., Philadelphia, Pa.

the liquid serum should never occupy more than one-half the volume of the container. It is essential to establish a relationship between the volume of serum and the evaporating surface; the depth of the serum at the time of freezing should not exceed 15 mm. By rotation the serum may be shelled in the container while freezing in order to increase the evaporating surface during the desiccation process. The dry ice bath is prepared by adding crushed dry ice to a liquid which does not freeze above  $-72^{\circ}\text{C}.$ ; alcohol or acetone is suitable. When the bath has become completely cooled, which is indicated by the absence of carbon dioxide bubbles, the vials containing the serum are submerged (Fig. 1A) and allowed to remain in the bath for approximately thirty minutes. After freezing,

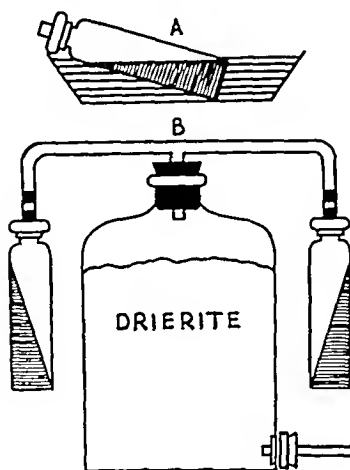


Fig. 1.—Schematic representation of the manner in which blood serum is (A) frozen and (B) dried.

the serum is quickly connected by means of the rubber stopper to the Wouff Bottle, which has been equipped with tight rubber stoppers and T-tube connections (glass) for the serum vials (Fig. 1B). The Wouff Bottle contains approximately 4 pounds of regenerated "Drierite." Simultaneously with the attachment or before, the vacuum pump is started. If the system is free from leaks, the moisture in the frozen serum will sublime to the chemical desiccant. As the moisture evaporates from the surface of the frozen serum, the removal of heat by vaporization is sufficient to retain the serum in the frozen state throughout the period of desiccation. Frost collects on the outside of the containers within seven minutes. The use of the stopcock grease on all connections was found desirable; first, to secure high vacuum throughout the system, and second, to facilitate the rapid attachment of the vials to the Wouff Bottle. The vacuum pump is allowed to maintain the vacuum of the system for a total of eighteen hours to insure complete drying. At the end of this period the metal sealing bands surrounding the necks of the rubber bottle are then compressed tightly. The rubber connections to the serum bottle are then severed from the and sealed in a vacuum is stored in a cold room until the time of feeding. Before making use of the process of freezing and drying of blood sera for the purpose of preservation, it was first necessary to determine whether the anti-

rachitic potency of serum was altered by this procedure. A number of sera were assayed immediately after the sample was taken and tested again after various periods of storage in the dried stage. No significant change of the vitamin D content was noticed even after one year of storage.

### OBSERVATIONS

Charts 1 to 6 indicate the daily doses of vitamin D administered and the concentration of vitamin D found in the blood serums at different times during and after treatment of each of six patients.

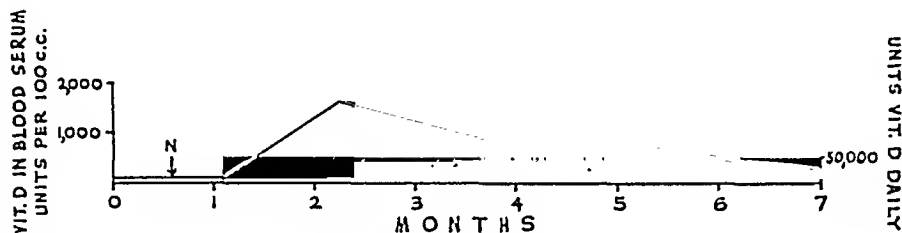


Chart 1.—Vitamin D level in blood serum of W. K. during oral administration of 50,000 units of vitamin D daily. N indicates the average vitamin D level in normal human blood serum (110 units per 100 c.c.)

In all charts the line indicates the vitamin D level in blood serum and the solid black portion indicates dosage.

CASE 1, Chart 1. W. K., white female, 45 years of age, was given 50,000 units of viosterol daily for six months. Due to an insufficient amount of sample, an incomplete assay was made of the blood serum obtained before the treatment was started. It was assumed, however, for making the chart, that the initial concentration of vitamin D in the blood serum was around 110 U.S.P. units per 100 c.c., corresponding to that found in other untreated persons previously reported. In the sample of serum obtained thirty-five days after the start of treatment, the concentration of vitamin D was 1,650 units per 100 c.c. With the same dosage continued, the concentration of vitamin D in the serum progressively decreased to 830, 660, and 330 units per 100 c.c., at two and one-half months, four months, and six months, respectively, after the start of treatment.

CASE 2, Chart 2. A. H. S., white male, 76 years of age, was given 150,000 units of crystalline vitamin D<sub>2</sub> daily during two and one-half months. In one month the concentration of vitamin D in his blood serum rose to 6,600 U.S.P. units per 100 c.c., remained constant eighteen days, and then decreased to 4,400 units per 100 c.c. before the treatment was discontinued. Two and a half months later the concentration of vitamin D in the blood serum had decreased to 1,700 units per 100 c.c. No further blood samples were taken thereafter for assay.

CASE 3, Chart 3. A. M., colored female, 43 years of age, took 180,000 units of vitamin D<sub>2</sub> daily for thirty-one days, and then discontinued treatment for eleven days. She then took 240,000 units of the same preparation daily for twenty-nine days. During the first period of medication the concentration of vitamin D in her blood serum rose to 3,300 units per 100 c.c., but fell to 1,600 units per 100 c.c. at the end of the eleven-day period without medication. At the end of the second period of medication the concentration again was 3,300 units per 100 c.c. Subsequently, the concentration of vitamin D in the blood serum fell to 1,600 units per 100 c.c. at the end of one month, to 800 at the end of two months, and to 330 at three and one-half months after the treatment was stopped.

CASE 4, Chart 4. H. R., colored female, 30 years of age, was given 200,000 units of viosterol daily for five days, then 300,000 units for twelve days, and 400,000 units daily thereafter for four months. The vitamin D in her blood serum increased rapidly to a concentration of 13,000 units per 100 c.c., two and one-half months after the treatment was started. Thereafter the concentration decreased, falling to around 8,000 units per 100 c.c.

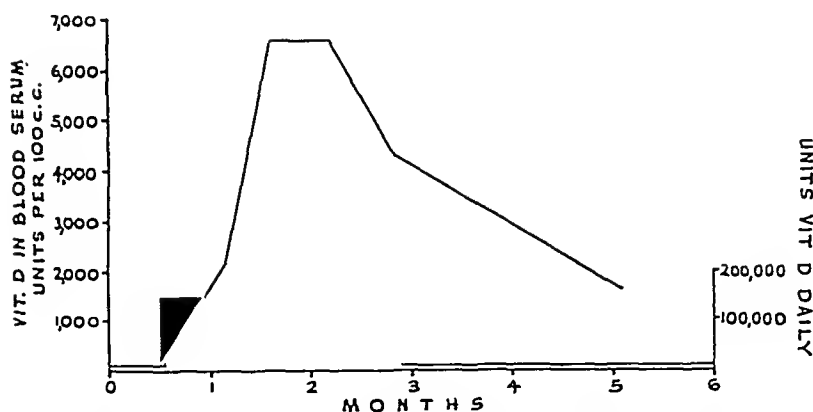


Chart 2.—Vitamin D level in blood serum of A. H. S. after oral administration of 150,000 units of vitamin D daily.

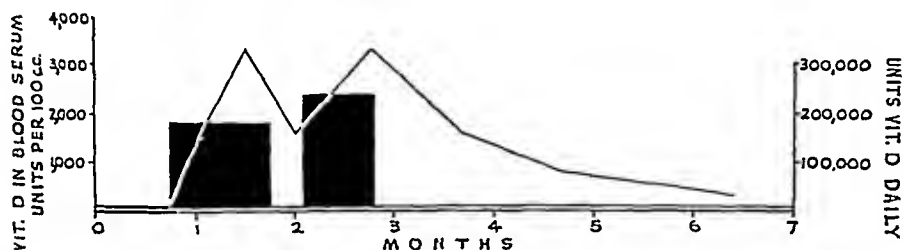


Chart 3.—Vitamin D level in blood serum of A. M. after oral administration of 180,000 to 240,000 units of vitamin D daily.

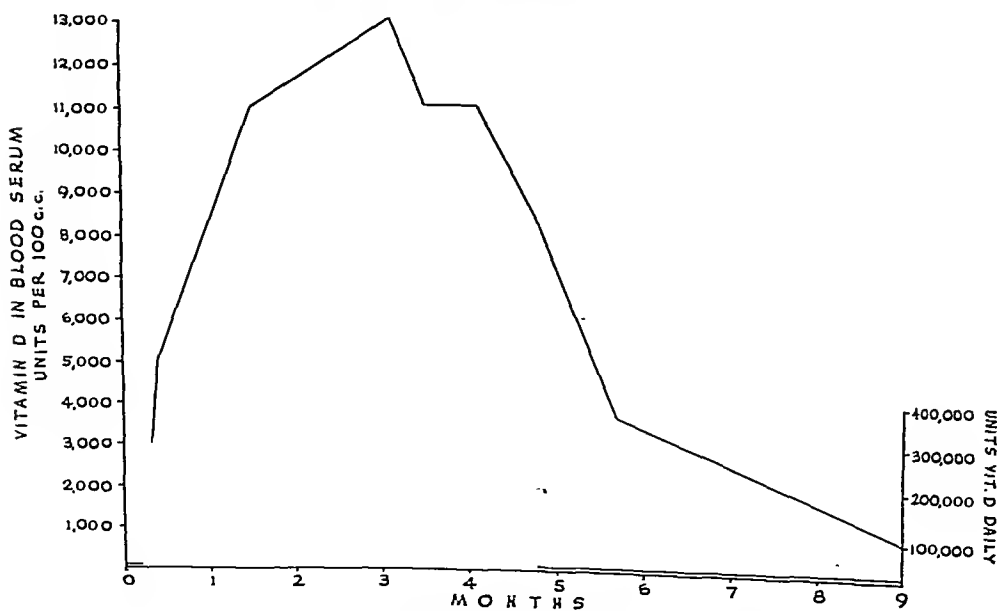


Chart 4.—Vitamin D level in blood serum of H. R. after oral administration of 400,000 units of vitamin D daily.

at the time the treatment was discontinued. After this date the concentration decreased to 5,500 units per 100 c.c. in one month, and to 330 units per 100 c.c. in four months.

CASE 5, Chart 5. E. B., white female, 36 years of age, was given viosterol in doses as follows: 150,000 units daily for three days, 300,000 units daily for seven days, 400,000 units daily for fourteen days, and 500,000 units daily for eighteen days. Thirst and polyuria developed, and the daily dose was decreased to 400,000 units, which she took during the following ten days. She then developed an infection of the respiratory tract and medication was stopped for five weeks. After this interval she again was given 400,000 units of the same preparation daily for forty days; no unfavorable symptoms developed. During the first period of medication the concentration of vitamin D in the blood serum increased to over 9,000 units

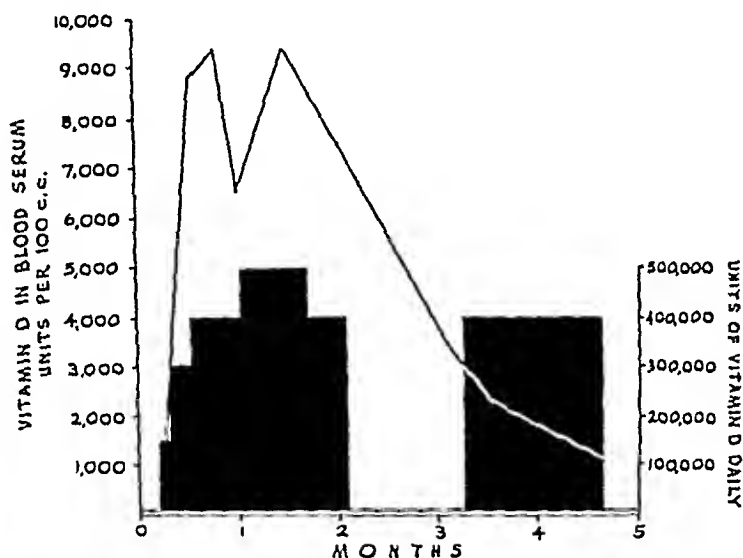


Chart 5.—Vitamin D level in blood serum of E. B. after oral administration of 150,000 to 500,000 units of vitamin D daily.

per 100 c.c., but decreased to 6,600 units per 100 c.c. in the sample taken at the end of the first period when 400,000 units were taken daily. During the period when 500,000 units were taken daily, the concentration again rose to above 9,000 units per 100 c.c. In the sample obtained after thirty days without medication, the concentration of vitamin D in the serum had fallen to 3,300 units per 100 c.c., and this concentration continued to decrease in spite of the resumption of daily doses of 400,000 units. Since this patient lived at home, it is, of course, possible that she did not take the medicine as prescribed, although she claimed that she had taken it faithfully.

CASE 6, Chart 6. G. B., white male, 54 years of age, was given two preparations of vitamin D in three different periods. In the first period viosterol was given daily as follows: 200,000 units for four days; 300,000 units for ten days; 400,000 units for seven days; 500,000 units for fourteen days; and 450,000 units for five days. Because of the development of thirst, polyuria, and slight nausea, medication was stopped. Seventeen days later he was given crystalline vitamin D<sub>2</sub>, 450,000 units daily for ten days. Nausea returned and medication was stopped for six months. In a third period viosterol was given in smaller doses: 50,000 units daily for eighteen days; 150,000 units for twenty-five days; and 200,000 units daily for five months. In Chart 6 are represented the changes in concentration of vitamin D in the blood serum during these successive periods with and without medication. In the first period the concentration rose to 13,200 units per 100 c.c., and then fell to 6,600 units per 100 c.c. at the end of the first rest period of seventeen days. During the second period, ten days, the concentration of vitamin D in the serum fell to 4,400 units per 100 c.c., although the patient again developed symptoms of intoxication. During the long rest period the con-



centration of vitamin D in the serum gradually fell to 330 units per 100 c.c. During the third period of medication the concentration of vitamin D in the blood serum rose to 4,400 units per 100 c.c., and then gradually decreased again in spite of the continued constant daily dose of viosterol.

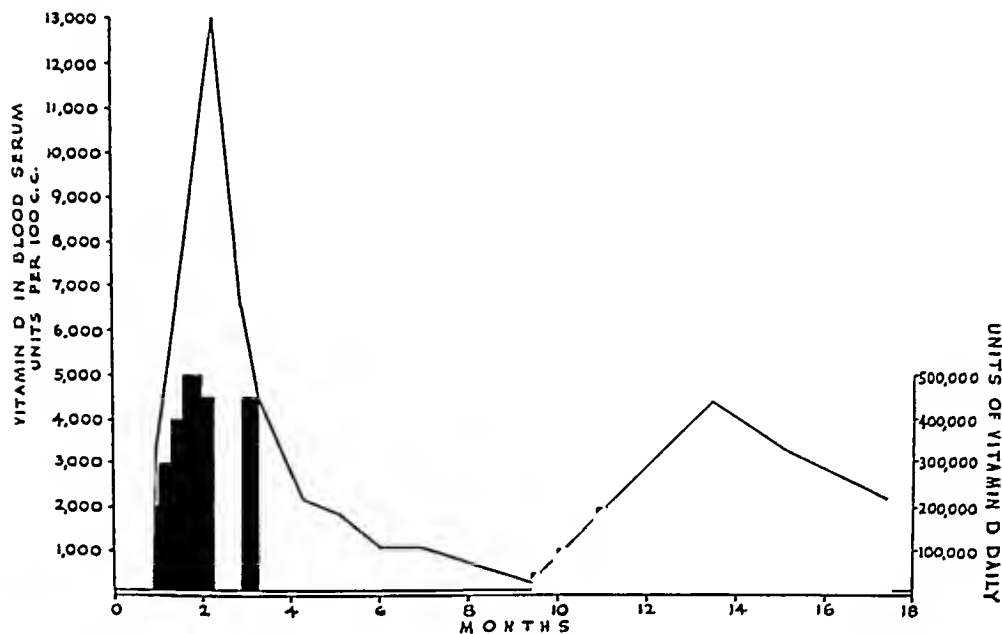


Chart 6.—Vitamin D level in blood serum of G. B. after oral administration of 200,000 to 500,000 units of vitamin D daily.

#### DISCUSSION

Prolonged oral administration of large doses of vitamin D (50,000 to 500,000 International units daily) to adult patients led to an increase in the antirachitic potency of their blood serum. The highest potency observed was more than one hundred times that of normal human blood serum. Such a concentration of vitamin D in the serum, equal to that of cod-liver oil, was tolerated by patient H. R. for more than two and a half months without any symptoms of intoxication, but another patient (G. B.) complained of thirst, polyuria, and nausea when a similar level of vitamin D in his serum was reached. The concentration of vitamin D in the serum was roughly proportional to the daily dose of vitamin D administered. This fact is demonstrated by Charts 1 to 6, previously discussed, and by data in Table I. Calculations of the vitamin D content of the entire volume of blood serum at the time when the concentration was at its maximum were based on the concentration of vitamin D per 100 c.c. of serum, the body weight, the blood volume (assumed to be  $\frac{1}{12}$  the body weight), and the per cent volume of cells in the blood (determined by centrifugation of a sample of heparinized blood, obtained at the same time as the sample used for serum vitamin D determination). In Table I are listed the amounts of vitamin D thus calculated to be present in the entire blood serum of each of six patients, and the daily dose of vitamin D each was taking when the concentration of vitamin D reached the highest level in the serum. In three patients (W. K., H. R., and G. B.) the maximal accumulation of vitamin D determined in the entire blood

serum corresponded closely to the amount that was being ingested daily. Four charts (1, 2, 4, and 6) suggest, however, that the maximal concentration of vitamin D in the serum was not maintained throughout the period during which the administration of large daily doses was continued. In Case 5, when the daily dose of 400,000 I. U. was discontinued, the concentration of vitamin D in the serum promptly decreased, as was to be expected. When the same dose was resumed, the concentration of vitamin D in the serum did not return to its previous level. Such findings might be interpreted to mean a diminishing absorption of the drug, increased excretion, or a more rapid rate of destruction of vitamin D in the body. Further studies would be required to determine which of these explanations is the most probable.

TABLE I

| PATIENT  | DAILY DOSES<br>(I.U.) | CALCULATED AMOUNT OF<br>VITAMIN D IN ENTIRE BLOOD<br>SERUM OF PATIENT<br>(I.U.) |
|----------|-----------------------|---|
| W. K.    | 50,000                | 54,000  |
| A. H. S. | 150,000               | 220,000   |
| A. M.    | 180,000               | 110,000   |
| H. R.    | 400,000               | 350,000   |
| E. B.    | 400,000               | 186,000   |
| G. B.    | 450,000               | 460,000   |

In most of the blood samples drawn from these patients for assay of the vitamin D in the serum, the distribution of inorganic and organic acid-soluble phosphorus in the serum and cells also was determined.<sup>9</sup> Although these data are not reported, it is germane to note here that the changes in distribution of phosphorus, such as were found in the bloods of these patients during their courses of treatment, were only slight and of doubtful significance. These investigations were made because of earlier findings that in some conditions vitamin D therapy brought about large changes in the concentration of both inorganic and organic phosphates in the blood. In rachitic animals with low concentrations of inorganic phosphorus in the blood serum and of phosphoric esters in the blood cells, the distribution of phosphorus in the blood became normal following the administration of small curative doses of vitamin D<sup>8-10</sup>. In normal rabbits the administration of large doses of irradiated ergosterol led to great increases of inorganic phosphorus in the whole blood and of organic acid-soluble phosphorus in the blood cells,<sup>11</sup> mainly accounted for in the diphosphoglycerate fraction.<sup>12</sup> The doses of irradiated ergosterol, which provoked such changes in the distribution of phosphorus in the blood of rabbits, were, however, much larger in proportion to body weight than the doses administered to the patients here reported. Moreover, the marked chemical changes of the bloods of rabbits were accompanied by definite signs of intoxication, such as loss of weight and hyperazotemia. No attempt was made to increase the doses of vitamin D given the patients to amounts causing more than very mild symptoms of intoxication. It is noteworthy that the concentration of vitamin D in the blood may be increased to many times the normal level without much, if any, change in the distribution of phosphorus in the blood.

\*With the cooperation of Dr. S. Rapoport.

## SUMMARY

A method of freezing and drying blood serum, which permits preservation and concentration of the serum without alteration of its vitamin D content, is described.

Data are reported on the vitamin D content of the blood serum of six adult patients during and after periods of taking large doses of vitamin D (50,000 to 500,000 International units daily).

During periods in which the daily high dose of vitamin D was constant, the concentration of vitamin D in the blood serum increased rapidly to a maximal level, which was roughly proportional to the daily dose. After doses of 400,000 to 500,000 units daily the concentration of vitamin D in the serum rose to between 9,000 and 13,000 I. U. per 100 c.c.; in other words, to an antirachitic potency equal to that of cod-liver oil. Few toxic symptoms were observed in the patients who took such doses of vitamin D.

During periods in which the daily high dose of vitamin D was constant there appeared to be a tendency for the concentration of vitamin D in the serum to decrease somewhat after attaining a maximal concentration.

After abrupt withdrawal of the high daily doses of vitamin D from three to six months elapsed before the concentration of vitamin D in the blood serum fell to a normal level.

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# DEPARTMENT OF ABSTRACTS AND REVIEWS

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ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

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**BLOOD:** Blood Cells in Healthy Young Infants, Washburn, A. H. *Am. J. Dis. Child.* 62: 530, 1941.

A description is given of the postnatal readjustments in the red blood cell values of 15 healthy infants during the first ten to eighteen weeks of life.

The simple explanation advanced for the observed changes is based on the comparison of the weekly values for the red blood cell count, the reticulocyte count, and the body weight for each individual baby. The comparison led to the conclusion that the decrease in the red blood cell count, the hemoglobin content, and the volume of packed cells in the first six to nine weeks of life is dependent largely on two factors.

The first factor is the rapid increase in total body mass. This appears to involve a similar rate of increase in total blood volume, so that the ensuing dilution results in fewer red blood cells per cubic millimeter of blood.

The second factor is the abrupt decrease in reticulocytes, indicating a sudden decrease in the rate of production of red blood cells. In most babies the rate of production between the ages of 1 week and 5 or 6 weeks appears to be too low for maintenance of the red blood cell level in the blood, especially in the presence of an increasing plasma volume.

The decrease in the number of red blood cells and in the amount of hemoglobin per cubic millimeter of blood results in a rise in the reticulocyte percentage at about the fifth to eighth week. The sharpness and the persistence of the rise in different babies vary, roughly, with the rapidity of weight gain and the speed of decrease in the red blood cell count.

In most babies the red blood cell count starts to increase between the sixth and the tenth week of life and continues to rise until it reaches a level of between 4,500,000 and 5,500,000 at about 16 weeks of age. Meanwhile, the number of reticulocytes decreases to approximately 1.0 per cent.

It seems probable that the period of postnatal adjustment of the red blood cells for most healthy babies extends over the first fifteen to twenty weeks of life.

**LEUKEMIA,** Erythroblastic, Stransky, E., and Quintos, F. N. *Am. J. Dis. Child.* 62: 577, 1941.

The case of a girl, aged 2 years 3 months with chronic myelogenous leucemia is presented. The patient died thirty-one hours after admission to the hospital. The observations on the blood and the bone marrow, as well as the histologic sections, were characteristic of chronic myelogenous leucemia. The most striking feature in the case was the marked erythroblastosis. There were 61,000 nucleated red blood cells per cubic millimeter in the peripheral blood and more than 280,000 in the bone marrow. The majority of the nucleated red blood cells were normoblasts. The clinical diagnosis was chronic myelogenous leucemia, and the hematologic diagnosis was severe erythroblastic anemia with chronic myelogenous leucemia; the final diagnosis in the case was erythroblastic leucemia. The similarity between the data in this case and those in cases of the so-called erythroleucoblastic type of hydrops foetalis congenitus is discussed. The unknown cause of chronic myelogenous leucemia may stimulate not only the granulocytopoietic tissue but, exceptionally, the erythropoietic tissue of the bone marrow.

**BLOOD LOSS, Response of Normal Subjects to, Ebert, R. V., Stead, E. A., and Gibson, J. G.** *Il. Arch. Int. Med.* 68: 578, 1911.

Seven hundred and sixty to 1,220 c.c. of blood (15.5 to 19.7 per cent of the total blood volume as determined by the plasma volume and the hematocrit reading) were removed from 6 normal persons in six to thirteen minutes. In 5 of the 6 there developed collapse, characterized by weakness, nausea, blurred vision, pallor, sweating, and fall in arterial pressure. Before the onset of collapse the heart rate increased from 14 to 30 beats per minute. At the height of collapse the heart rate became slow, ranging between 36 and 40 beats per minute.

The plasma volume began to increase immediately after hemorrhage and continued to increase for the next forty-eight to seventy-two hours. At the end of seventy-two hours it was approximately equal to the plasma volume before hemorrhage plus the volume of red blood cells removed.

During the first two hours after venesection the plasma volume was increased by protein-poor fluid, so that the serum protein concentration decreased. Thereafter, fluid and protein were added to the plasma at the same time, and the serum protein concentration remained unchanged, though the total circulating protein increased.

At the end of seventy-two hours approximately one-fourth of the plasma protein was protein which had been added to the plasma after venesection. Loss of blood acted as a physiologic stimulus for the production of normal protein, and the addition of the new protein did not change the proportions of the albumin and the various globulin fractions.

When physiologic solution of sodium chloride was given intravenously immediately after hemorrhage, it was not retained in the blood stream in sufficient quantity to restore the plasma volume to normal.

After hemorrhage the blood volume was not restored to normal until new plasma protein had been added to the circulation.

Hematocrit readings in these experiments accurately reflected the direction of a change in plasma volume.

**NEPHROSIS: Acacia in the Treatment of the Nephrotic Syndrome, Goudsmit, A., Binger, M. W., and Keith, N. M.** *Arch. Int. Med.* 68: 513, 1941.

Intravenous injection of acacia into 4 patients who had the nephrotic type of edema was followed by marked increases in urinary excretion of water and of chloride (expressed in terms of the sodium salt). This increased excretion of sodium chloride and of water brings about a reversal of conditions which is significant and beneficial, considering the excretion by untreated patients. Neither increases in the volume of the circulating blood nor changes of colloid osmotic pressure appear to account for this change in renal function. Experiments on animals have also demonstrated this increased excretion of chloride after intravenous administration of acacia. It is suggested that an analogous effect might well be responsible for the diuretic response of these patients.

**FLUIDS, BODY, Suggested Modification of Mandelbaum's Method of Examining, for Cells, Tannhauser, S.** *Arch. Path.* 32: 450, 1941.

The fluid (pleural or ascitic) is collected with addition of an appropriate amount of an anticoagulant (sodium citrate or potassium or sodium oxalate). After centrifuging the mixture in a large tube with a flat round bottom, a suitable amount of oxalated or citrated plasma is added; double the quantity of the remaining centrifugate has been found appropriate. If, for instance, an oxalated plasma, obtained by adding 0.4 c.c. of 4 per cent potassium oxalate to each 10 c.c. of blood, is used, 10 drops of a 0.5 per cent solution of calcium chloride for each cubic centimeter of plasma will induce clotting. It is important to add foreign plasma and not to rely on the clotting properties of the body fluid, since often this will not clot, owing to previous spontaneous defibrinization. The centrifugate is thoroughly

mixed with the plasma, and calcium chloride is added. In a short time clotting starts and encloses all cells present in the centrifugate. After retraction the clot is transferred to a 10 per cent solution of formaldehyde or Zenker's fluid for fixation, and its further treatment is that of any other specimen, with the fibrin of the clot holding the cells together in a satisfactory way.

When oxalated or citrated plasma is not available, a few centimeters of freshly obtained venous blood may also be used to advantage.

The particles of malignant growth stand out well from the blood clot. In case an examination of the cellular content of the fluid is desired besides examination for cells or particles of malignant growth, that has to be done on a separate smear before addition of the blood.

This modification of the method of Mandelbaum may also be used when examination of sputum for malignant cells is to be made and paraffin embedding of the whole sputum is desired. The procedure then differs somewhat from that described:

In order to prevent disintegration of the cells by the action of the always present bacteria, the sputum is directly collected in a 10 per cent solution of formaldehyde. Usually the mucous sputum balls are dissolved or precipitated in small fragments by the formaldehyde (except when the sputum is highly albuminous; it then is more coherent after fixation with a solution of formaldehyde alone). After fixation of the sputum for twenty-four hours, the particles are centrifuged and washed twice with distilled water; a suitable amount of plasma with calcium chloride solution is then added. The resulting clot is again transferred to a solution of formaldehyde or Zenker's solution for fixation, washed and dehydrated in the graduated alcohols as already described.

#### FORENSIC MEDICINE: Significance of Dextrose and Non Dextrose Reducing Substances in Postmortem Blood, Hill, E. V. Arch. Path. 32: 452, 1941.

Post-mortem samples of blood for determinations of dextrose should be removed from the left side of the heart if significant errors due to the post-mortem diffusion of dextrose from the liver to the right side of the heart are to be avoided. The dextrose responsible for the post-mortem rise in dextrose on the right side of the heart is liberated by glycogenolysis occurring in the liver. Fasting animals or cadavers presenting marked hepatic damage do not show this post-mortem rise in dextrose.

Glycolysis occurring after death causes a progressive lowering of the dextrose content of the heart's blood. Intravascular and in vitro glycolysis occur at approximately the same rate and are not influenced by clotting of the blood.

The rate of glycolysis was studied in vitro at various temperatures, and the temperature coefficient of the reaction was determined.

The dextrose and nondextrose reducing substances were determined in 73 medicolegal and hospital cases. Significant quantities of residual dextrose were present in the majority of cases of asphyxia, shock, acute coronary closure, rapidly developing anoxemia, increasing intracranial pressure, and fluoride poisoning, indicating that agonal hyperglycemia was present.

For significant results in cases in which hypoglycemia is suspected, specimens of blood for analysis should be taken within two hours after death.

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## CLINICAL AND EXPERIMENTAL

### PRODUCTION OF PYROGEN BY SOME BACTERIA\*

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#### HISTORICAL REVIEW

IN 1911 Hort and Penfold showed that the intravenous injection of freshly distilled water caused no symptoms in animals and in men, while the intravenous injection of the same water, after it had been incubated in unsterile vessels, caused a febrile reaction. Since the fever-producing principle was still present after the water had been passed through a Berkefeld filter, these authors postulated that this principle, provisionally called pyrogen, was a product associated with bacteria but was not a part of the bacterial bodies themselves.

In 1923 Siebert, studying the febrile reaction following the intravenous administration of distilled water in rabbits, confirmed the findings of Hort and Penfold, and showed conclusively the bacterial origin of pyrogen. With Bourn, Siebert in 1925 studied 25 strains of bacteria isolated from distilled waters and reported that those responsible for the production of mild fevers were related to the Jordan group X of river-water bacteria, those producing severe fevers to group XI, while the nonpyrogenic bacteria fell into group XIII, which were chromogenic. According to a more recent classification (Bergey, 1930), these organisms would all be in the family *Bacteriaceae*, with the strong and mild pyrogen producers belonging to the genera *Achromobacter* and *Escherichia*, and the nonpyrogenic organisms to several genera of the tribe *Chromobacterieae*.

Banks in 1934 reported that *Pseudomonas ureae* and *scissa*, two species of the genus *Pseudomonas* which he isolated from surface waters, were pyrogenic. Apparently not familiar with the more extensive bacteriologic work of Bourn and Siebert, he stated categorically that these two organisms were specific pro-

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ducers of pyrogen, and that pathogens, as represented by *Staphylococcus aureus*, and nonpathogens, as represented by *Bacillus subtilis*, were productive of a delayed febrile response and, therefore, could not be classified as pyrogen producers. Banks's work, while containing many conclusions now found untenable, added one more genus to those found by Bourn and Siebert as pyrogen producers and refuted Siebert's impression that pyrogenicity was associated with achromogenicity.

Thus far the study of pyrogen production has been confined to the bacteria found in waters. However, the striking parallelism between the symptomatology of a pyrogenic reaction from "reactive" waters, and that caused by the intravenous injection of typhoid vaccine, or of aëcia and sera made "reactive" by the implantation of other strains of bacteria, indicates the close kinship of these fever-producing principles. This kinship is made still closer by the similarity of their filtration characteristics as shown by Co Tui, Benaglia, Ruggiero, and Yates.

It thus appears that pyrogen production may be much more widespread than was first embraced in the concepts of Siebert and Banks. The present work is an attempt to determine how universal this phenomenon is.

#### EXPERIMENTAL PROCEDURE

Eighteen strains of microorganisms belonging to different genera, tribes, and families were investigated. They were prepared for tests in three different ways.

In the first series 12 bacterial strains grown in initially pyrogen-free broths were used. The cultures were incubated for forty-eight hours at their particular optimum temperatures, and then centrifuged at 4,000 r.p.m. for forty-five minutes in an angle centrifuge. The supernatant fluid was filtered through a Berkefeld "W" filter, and the sterile filtrate was tested. These tests may be considered primary tests, since the immediate media in which the organisms grew were used.

The second series consisted of bacteria which were more convenient to grow on slants or in Kolle flasks. The colonies were washed out with a few cubic centimeters of pyrogen-free physiologic saline solution containing 0.4 per cent phenol, inactivated for forty-five minutes by heat. The suspension was filtered as in the case of broth cultures, and the filtrate was tested. These tests may likewise be considered primary tests, since whatever pyrogen was present in the colonies would presumably go into solution in the suspending media. Four organisms were tested in this way.

The third series consisted of nine organisms tested in the form of vaccine made with pyrogen-free water. The first three were highly pathogenic organisms; namely, *Vibrio comma*, *Neisseria intracellularis*, and *Pasteurella pestis*. These organisms were prepared in sets of two; one set by a biological firm and the other by the bacteriologic department of the university. The other six organisms in this series had been tested in series one, so that the tests in this case serve merely as a check. All these vaccines were passed through a Berkefeld filter, as in the case of the other two series and the cell-free filtrate used. The



tests in this series may be thought of as secondary tests, since the media tested were not in immediate contact with the living bacteria. While a positive finding may be considered significant, a negative finding may only mean that the pyrogen produced may be so slight as to be lost in the process of making the vaccines.

A positive pyrogenic reaction in the dog, as reported in our previous communications, consists of a leucopenia thirty to forty-five minutes after the intravenous injection of the pyrogenic material; a rise of temperature which begins to be perceptible thirty minutes after injection, reaching a peak in from two to four hours and returning to normal in four or five hours. In mildly severe reactions there are prostration and gastrointestinal symptoms, such as vomiting and diarrhea, both of which may contain blood. These symptoms are comparable to those in men.

The dog is used throughout this work not only because of the consistency of its temperature curve, but also because the constancy of the leucopenia and the frequent appearance of the secondary symptoms of prostration and gastrointestinal disturbances serve as an added check.

That this pyrogenic reaction is not due to nucleic acid is shown in three experiments in which the intravenous injection of 1, 3, and 5 mg. of pure nucleic acid, prepared by Dr. P. A. Levene, into each of three dogs failed to provoke a similar response.

#### DISCUSSION OF RESULTS

Table I summarizes the results of this investigation. The first four organisms have not been classified as to either species or genera, but belong to the family of the Baeteriaceae of the tribes Achromobaeterieae and Chromobacterieae. W14B and W14C were isolated from well water, and 31y and 2WS from pond water. Their best growth was obtained at 20° C. They were gram-negative bacilli, varying in shape from minute bacillary forms in the case of W14B to a large coccoid form in the case of W14C. 31y was a small bacillus exhibiting chromogenic properties and imparting to the broth and agar preparations a light yellow color. 2WS was the largest bacillus of the group and often grew in long chains.

All these four organisms were included here to show the similarity of the pyrogenic reaction elicited by the injection of their products and that elicited by the injection of the products of microorganisms of other groups.

Each item listed in Table I has been selected from a series of at least five experiments as the one best representative of that series.

A study of the table shows a number of interesting facts. The first 13 organisms listed in Table I are all pyrogen producers, 9 of them not being members of the river-water group. Four of these, namely, *E. typhi*, *Streptococcus pyogenes*, *Vibrio comma*, and *N. intracellularis* are pathogens. Two others, *E. coli* and *Staphylococcus aureus*, are facultative saprophytes. It will also be seen that it takes different amounts of the broth filtrate to produce a reaction of an intensity similar to the range of reactions common to the pyrogen producers in the table, the dose ranging from 1 c.c. to 15 c.c. While this may be interpreted to mean that some microorganisms are better pyrogen producers

TABLE I

| ORGANISM                      | FILTRATE TESTED | AMOUNT USED (C.C.) | CONC. PER C.C. VACCINE IN BILLIONS | DOG WEIGHT (KG.) | W.B.C. DROP (THOUSANDS) | CHANGES IN TEMP. (° F.) | SYMPTOMS                                    |
|-------------------------------|-----------------|--------------------|------------------------------------|------------------|-------------------------|-------------------------|---|
| W14B                          | B               | 10                 |                                    | 16               | 14.5-1.3                | 101-101                 | Shivering, emesis and bloody stool          |
|                               | V               | 5                  | 20                                 | 9                | 19.6-3.0                | 102.4-104.8             | None  |
| W14C                          | B               | 10                 |                                    | 16               | 12.2-3.2                | 100.2-103.2             | Violent shivering, emesis, and bloody stool |
|                               | V               | 1                  | 10                                 | 9                | 22.9-4.6                | 102-105                 | Violent shivering                           |
| 31y                           | B               | 5                  |                                    | 11               | 17.6-4.7                | 101.6-103.6             | Violent shivering and emesis                |
|                               | V               | 5                  | 20                                 | 11               | 15.2-2.2                | 101.6-103.6             | Shivering                                   |
| 2WS                           | B               | 5                  |                                    | 17               | 15.5-4.2                | 101.6-106.4             | Shivering, emesis, and defecation           |
|                               | V               | 15                 | 20                                 | 9                | 15.4-5.2                | 101.4-104.6             | Shivering and emesis                        |
| Eseherichia coli              | B               | 1                  |                                    | 11               | 15.5-2.1                | 101.6-103.6             | Violent shivering and emesis                |
|                               | V               | 1                  | 10                                 | 13               | 21.1-4.6                | 101.4-103.6             | Violent shivering                           |
| Eberthella typhi              | B               | 1                  |                                    | 11               | 11.9-2.9                | 102-104                 | Shivering, emesis, and defecation           |
|                               | V               | 1                  | 10                                 | 11               | 20.7-4.1                | 101.8-104.6             | Shivering, emesis, and defecation           |
| Streptococcus pyogenes        | B               | 7                  |                                    | 8                | 14.8-7.6                | 101.8-103.6             | Shivering, emesis, and bloody stool         |
| Proteus vulgaris              | B               | 1                  |                                    | 13.5             | 11.9-2.9                | 102-104                 | Shivering, emesis, and defecation           |
| Staphylococcus aureus         | B               | 10                 |                                    | 13               | 38.6-25.0               | 102.8-104               | Shivering, emesis, and defecation           |
| Vibrio comma                  | V               | 1                  | 3                                  | 9                | 16.9-4.3                | 101.8-106               | Emesis, shivering and defecation            |
| Neisseria intracellularis     | V               | 8                  | 1                                  | 16               | 3.7-0.8                 | 101.4-104.6             | Emesis and defecation                       |
| Saccharomyces Pink wild yeast | B               | 15                 |                                    | 16               | 22.5-6.9                | 100.6-103.0             | None  |

B - Broth.  
V - Vaccine.  
S - Suspension.  
Toxin broths heat inactivated.

All sterile broth controls used in culturing organisms gave no reactions on intravenous injections in dogs, i.e., no changes in temperature, white blood cell count, or any systemic symptoms.

TABLE I—CONT'D

| ORGANISM                           | FILTRATE TESTED | AMOUNT USED (C.C.) | CONC. PER C.C. VACCINE IN MILLIONS | DOG WEIGHT (KG.) | W.B.C. DROP (THOUSANDS) | CHANGES IN TEMP. (° F.) | SYMPTOMS                                |
|------------------------------------|-----------------|--------------------|------------------------------------|------------------|-------------------------|-------------------------|---|
| <i>Bacillus subtilis</i>           | B               | 5                  |                                    | 16               | 31.0-3.5                | 102.8-105.6             | Defecation with blood, animal depressed |
| <i>Corynebacterium diphtheriae</i> | B               | 17                 |                                    | 21               | 10.2-9.5                | 101.2-100.8             | None                                    |
|                                    | S               | 12                 |                                    | 12               | 16.3-16.4               | 101.6-101.4             | None                                    |
| <i>Pasteurella pestis</i>          | V               | 5                  | 5                                  | 9                | 16.6-13.5               | 100.8-100.8             | None                                    |
| <i>Pasteurella cuniculicida</i>    | S               | 12                 |                                    | 10               | 24.5-20.1               | 101.6-101.6             | None                                    |
| <i>Mycobacterium tuberculosis</i>  | S               | 15                 |                                    | 18               | 36.0-36.0               | 102.6-102.8             | None                                    |
| <i>Neisseria gonorrhoeae</i>       | S               | 17                 |                                    | 12               | 17.9-10.0               | 102.0-102.0             | None                                    |

than others, yet it could also mean that the rates of rapidity of their proliferation may be different. Unless bacterial counts are made of the cultures, this point cannot be settled.

In the case of the vaccines, however, where some quantitation has been used, the indication that some bacteria are better pyrogen producers than others is stronger. Thus of the 8 organisms tested in the vaccine form, it takes only three billion cells of *V. comma* to produce a cell-free vaccine filtrate which causes a temperature rise of 4.2° F., ten billion in the case of *E. coli* to produce a rise of 3.1° F., and ten billion of *E. typhi* to produce a rise of 2.8° F., while it takes 300 billion of 2WS to produce a rise of 3.5° F. These figures would seem to indicate that *V. comma* is the most efficient producer of pyrogen among these strains with *E. coli*, *E. typhi*, *N. intracellularis*, and W14C second and 2WS the least.

Of practical importance from the viewpoint of air contamination in the production of pyrogen in the preparation of medicaments and other chemical solutions is that *Proteus vulgaris* and *B. subtilis* are pyrogen producers.

It is also of particular significance that wild yeast produces pyrogen. This opens the field for further experimentation in group of yeast and molds.

The last five microorganisms in the table; namely, *C. diphtheriae*, *Pasteurella pestis*, *Pasteurella cuniculicida*, *M. tuberculosis* and the *gonorrhoeae* were according to these tests nonpyrogenic. All these were subjected to primary tests, except *Pasteurella pestis*, which was tested only in the vaccine form, using an amount equivalent to 25 billion cells. *Pasteurella pestis* is thus at best a weak pyrogen producer if at all. In all these negative tests it is interesting to note

TABLE II  
CLASS SCHIZOMYCETES  
ORDER EUBACTERIALES

| FAMILY           | TRIBE                 | GENUS           | ORGANISM             | PYROGEN PRODUCTION | INVESTIGATOR               |
|------------------|-----------------------|-----------------|----------------------|--------------------|----------------------------|
| Coccaceae        | Streptococcaceae      | Streptococcus   | pyogenes             | Positive           | Co Tui, Schrift, and Banks |
|                  | Neisseriaceae         | Neisseria       | gonorrhoeae          | Doubtful           |                            |
|                  | Neisseriaceae         | Neisseria       | intracellu-<br>laris | Positive           |                            |
|                  | Micrococcaceae        | Staphylococcus  | aureus               | Positive           |                            |
| Spirillaceae     |                       | Vibrio          | comma                | Positive           |                            |
| Bacteriaceae     | Chromobacteriaceae    | Serratia        | ruber                | Negative           | Bourn and Siebert          |
|                  | Chromobacteriaceae    | Flavobacterium  | radiatum             | Negative           | Bourn and Siebert          |
|                  | Chromobacteriaceae    | Flavobacterium  | ochraceum            | Negative           | Bourn and Siebert          |
|                  | Chromobacteriaceae    | Flavobacterium  | arborescens          | Negative           | Bourn and Siebert          |
|                  | Chromobacteriaceae    | Pseudomonas     | urea                 | Positive           | Banks                      |
|                  | Chromobacteriaceae    | Pseudomonas     | seissa               | Positive           | Banks                      |
|                  | Chromobacteriaceae    |                 | gly                  | Positive           |                            |
|                  | Achromobacteriaceae   | Achromobacter   | pinnatum             | Positive           | Bourn and Siebert          |
|                  | Achromobacteriaceae   | Achromobacter   | solitarium           | Positive           | Bourn and Siebert          |
|                  | Achromobacteriaceae   | Achromobacter   | candicans            | Positive           | Bourn and Siebert          |
|                  | Achromobacteriaceae   | Achromobacter   | refractans           | Positive           | Bourn and Siebert          |
|                  | Achromobacteriaceae   | Achromobacter   | tiogense             | Positive           | Bourn and Siebert          |
|                  | Achromobacteriaceae   | Achromobacter   | punctatum            | Positive           | Bourn and Siebert          |
|                  | Achromobacteriaceae   | Achromobacter   | lactium              | Positive           | Bourn and Siebert          |
|                  | Achromobacteriaceae   | Achromobacter   | W14B                 | Positive           | Bourn and Siebert          |
|                  | Achromobacteriaceae   | Achromobacter   | W14C                 | Positive           |                            |
|                  | Achromobacteriaceae   | Achromobacter   | 2WS                  | Positive           |                            |
|                  | Bacteriaceae          | Escherichia     | formica              | Positive           |                            |
|                  | Bacteriaceae          | Escherichia     | coli                 | Positive           |                            |
|                  | Bacteriaceae          | Proteus         | vulgaris             | Positive           |                            |
|                  | Bacteriaceae          | Eberthella      | typhi                | Positive           |                            |
|                  | Pasteurellaceae       | Pasteurella     | pestis               | Positive           |                            |
|                  | Pasteurellaceae       | Pasteurella     | cuniculicida         | Negative           |                            |
|                  |                       | Bacillus        | subtilis             | Positive           |                            |
| Bacillaceae      |                       |                 |                      |                    | Co Tui, Schrift, and Banks |
| Mycobacteriaceae | ORDER ACTINOMYCETALES |                 |                      |                    |                            |
|                  |                       | Mycobacterium   | tuberculosis         | Negative           |                            |
|                  |                       | Corynebacterium | diphtheriae          | Negative           |                            |

that the white blood cell count did not undergo any substantial change except in the case of the *N. gonorrhoeae* test in which there was a suggestive fall.

It is clear from these studies that the matter of pyrogen production is not associated with pathogenicity as first postulated by Banks or with chromogenicity as postulated by Siebert (1923), or with the property of taking or rejecting the Gram stain as suggested by Cecil.

In Table II are compiled all the bacteria tested for pyrogen production from data furnished in the work of Siebert, of Banks, and in our present work. It will be seen that the greatest number tested have been selected from the order of the Eubacteriales of the class Schizomycetes, with particular emphasis on the family *Bacteriaceae*. In addition to the three tribes of the family *Bacteriaceae* that Siebert found pyrogenic and to the one genus of the tribe *Chromobacterieae* by Banks, the present work has added four families of the order Eubacteriales, one family of the order Actinomycetales, embracing a total of eleven known genera and thirteen known species.

#### SUMMARY

In a study of pyrogen production by representatives of different orders, families, genera, and species of bacteria, it was found that this phenomenon is a more widespread one than has hitherto been envisaged. It is not confined to any one group, but occurs in a number of different groups. Nor is it associated with nucleic acid, with pathogenicity, chromogenicity, nor with the property of taking the Gram stain. The production of pyrogen by a member of the yeast group suggests that even higher forms than bacteria may possess this property of pyrogen production. How pyrogen is related to the various toxic substances isolated by various workers from different bacteria remains to be studied. This statement implies that chemically and immunologically pyrogen is still an unknown entity. It is interesting to note that in this work a physiologic criterion has been used for the study of a bacterial product, a criterion distinct from those in use heretofore, namely, death or the production of antibodies in experimental animals. It is quite conceivable that there may be some bacterial products which are neither lethal nor antigenic and are, nevertheless, physiologically potent.

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## INTESTINAL VASCULAR SCLEROSIS\*

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**I**NTESTINAL vascular sclerosis is a condition of the mesenteric blood vessels usually associated with old age and often accompanied by disordered bowel function.

*Pathology.*—Narrowing of the extramural and intramural mesenteric vessels is part of a more generalized systemic condition of degenerative vascular disease associated with senescence. From the pathologic viewpoint these changes consist chiefly of disruption or disappearance of the elastic lamina, fatty degeneration with subintimal proliferation, calcific deposition, and atrophy of the media. The end result is a narrowed, rigid vessel, which is unresponsive to the ever-changing circulatory requirements of the organs supplied. The most advanced disease is generally seen in the larger arteries, where actual widening of the lumen may occur. This is essentially due to weakening of the wall from degeneration of the media and elastica. In the smaller arteries, however, such as those seen in the intestinal submucosa, the most common change noted is a subintimal proliferation of connective tissue. This deposition of connective tissue is often accompanied by thickening of the elastica, both representing an adaptive overgrowth to bolster the overlying weakened media. Occasionally this process occurs in young people following systemic infections, but its prevalence in the older age groups appears to be associated with the biologic process of aging. Complete obliteration of the intramural intestinal vessels sometimes occurs and can be readily detected at necropsy by subjecting the opened bowel to transillumination.<sup>1</sup> They stand out as straight, tapering, white, bloodless, threadlike filaments. By careful section of selected areas one can often trace successive stages in the same vessel. In some instances narrow clefts form in the fibrous tissue which obliterates the vessel lumen. These clefts are lined by endothelial cells; they often contain erythrocytes and represent an attempt at recanalization. In this manner it appears quite possible that a fair blood supply may be maintained, though it is probably inadequate as compared with that carried by the normally patent vessel.

*Clinical Aspects.*—In mild, moderate, or recanalized forms of intestinal vascular sclerosis few or no symptoms may be present. In patients with the more advanced type of disease the chief clinical symptoms and signs are abdominal distention, constipation, indigestion, and abdominal cramps after eating. One or all may be present, depending upon the degree of vascular narrowing. They are most frequently encountered in the sixth and later decades of life, but they may be present earlier. The adage "a man is as old as his

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arteries" is applicable to the intestinal vessels. "Senescence" is a relative term, however, as Boas<sup>2</sup> has recently pointed out, varying considerably in different persons. Thus, relatively young people may have intestinal vascular sclerosis, and some old people are comparatively free of it (Humphrey,<sup>3</sup> Groddeck<sup>4</sup>). The former generally exhibit concomitant changes in the glomerular tufts and larger renal vessels with persistent hypertension, either because of a glomerular nephritis complicating some infectious disease or as the late phase of essential hypertension with arteriosclerosis. In older people, however, hypertension is not invariably present any more than in coronary sclerosis, or sclerosis of the cerebral arteries. Intestinal vascular sclerosis is essentially a focal manifestation of generalized arteriosclerosis. The thought has often occurred to us that the epigastric and other abdominal discomfort in coronary disease is often due to mesenteric sclerosis. The symptoms of which patients with mesenteric sclerosis complain are often regarded as being on a purely functional basis. This, however, is not the case, as can be shown by careful general physical examination and sigmoidoscopy. The changes noted by the latter method are distinctive and readily elicited by examination of the submucosal intestinal vessels. During life, with blood coursing through the vascular arterial branches, their relative thickness, elasticity, and general contour can be determined with the aid of a telescopic device and green color filter<sup>5</sup> (Wrattan X). Normally, the submucosal vessels can be seen as graceful, delicate, long, red, sinuous undulations, gradually tapering to their tips. Their elasticity can be determined to some extent by means of intermittent suction applied through the inflation tube of the sigmoidoscope. In this manner a transient period of negative pressure is created. Normal vessels, alternately redden and pale, correspond to the phases of negative and positive pressure. With advancing sclerosis and narrowing of the lumen the arteries become thicker, the undulations abrupt, tortuous, or corkscrew, and alternate suction and release produce no appreciable reddening and paling of the vessels. During the late or obliterative stage the vessels pursue a short straight course, terminating rather abruptly in white bloodless cords which do not respond to suction. Examination of the eye grounds is suggested as a supplementary procedure to sigmoidoscopy, for the retinal vessels parallel the intramural intestinal branches both as to size and extent of pathology.

The mechanism underlying the symptomatology in intestinal vascular sclerosis appears to be a transient or permanent ischemia with relative anoxemia. The bowel is a hollow muscular organ, with an exceedingly rich blood supply that must be adequately maintained for effective contractility and functioning of the digestive glands. Interference with the blood and oxygen requirements of the musculature produces spasm (i.e., muscle cramp) and later, atony. Barker<sup>6</sup> states that in case histories of 260 patients over 60 years of age, digestive symptoms were second highest in frequency. While many factors must be considered, such as inadequate mastication, there still remains a large group of persons whose symptomatology can only be explained by some factor incident to the aging process. Patients will often state that small amounts of easily digested food are better tolerated than large meals, an ob-

servation that many of our keen predecessors put to practical use in the treatment of elderly patients with digestive complaints. The reason is obvious, since easily digested foods in small amounts require less work on the part of the intestine for their assimilation than bulky or indigestible foods. The impaired blood supply under these circumstances may be adequate because the digestive requirements have been adapted to the functional capacity of the bowel.

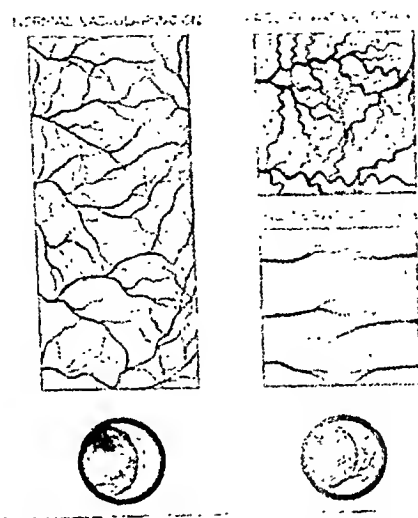


Fig. 1.—Arteriosclerosis. Mesenteric vessels thickened tortuous, or partially obliterated. Corresponds in individual cases to appearance of retinal vessels.

With advancing years, as the vascular changes progress, the intestinal musculature tends to lose its tone. This is particularly true in the colon where gas, which is normally present, is not passed readily and tends to accumulate with increasing atony of the wall, thus perpetuating a vicious cycle. The chief signs of intestinal atony are distention and constipation. In this connection it may be of interest to recall the relationship of distention to impaired gas diffusion through the intestinal wall. Normally, as pointed out by McIver, Redfield, Benedict,<sup>7</sup> and others, the intestinal wall serves as a semipermeable membrane, permitting diffusion of gases in either direction, i.e., from the intestinal lumen to the blood, or vice versa. Any interference in the free passage of gases may lead to flatulence (Dunn and Thompson).<sup>8</sup> Alvarez,<sup>9</sup> Kato,<sup>10</sup> Kader,<sup>11</sup> Schoen,<sup>12</sup> and others regard impaired circulation in the mesenteric vessels as one of the prime factors in the production of distention. Schoen stresses, in addition, the lowering in tone of the intestinal musculature. It is my impression that both factors are closely related to intestinal vascular sclerosis and are often the result of it. In rare instances extreme sclerosis of the mesenteric vessels may result in rupture, i.e., abdominal apoplexy. This subject has been recently reviewed by Crile and Newell,<sup>13</sup> who report ten cases from the literature in addition to their own. Sometimes complete occlusion of a main mesenteric arterial branch occurs with gangrene of the part of the intestine supplied. Here the process usually consists of a superimposed thrombosis in a markedly narrowed lumen.



The frequent occurrence of flatulence in patients with hypertension is well recognized, Kantor and Marks<sup>11</sup> reporting an incidence of over 50 per cent. The association of intestinal vascular sclerosis with long-standing hypertension is, in our experience, quite constant. In reviewing 1,000 necropsies at the Bronx Hospital from the standpoint of concomitant intestinal vascular sclerosis and generalized arteriosclerosis in the same person, agreement was found in all instances where advanced changes were present in the larger vessels. It may be stated that all intestinal necropsy specimens were viewed by means of the transilluminator.

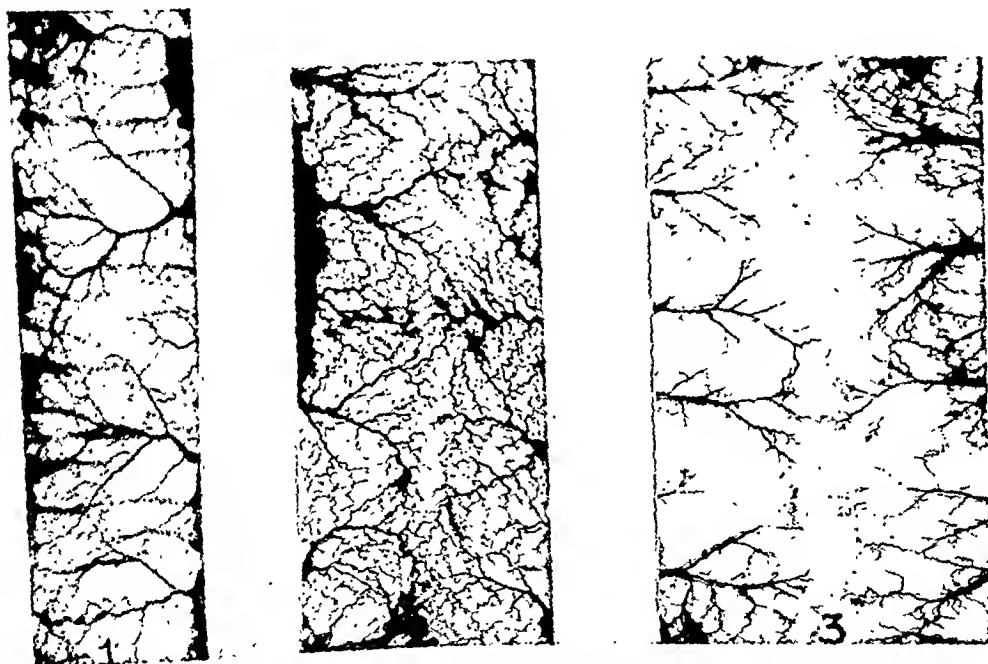


Fig. 2.—Intestinal transparencies. 1, Normal intramural vascularization. Note long, delicate, sinuous undulations with gradual tapering to finest branches which anastomose freely with adjacent vessels. 2, Proliferative phase. Vessels are thickened and tortuous. 3, Obliterative phase. Vessels are straightened and rigid with obliterated terminal arborizations. 2 and 3 are from Case 2 (No. 9048).

Interference with diffusion of gases may be due to a thickened intima or impaired circulation of blood in the intestinal arterioles. In cirrhosis of the liver or failure of the cardiac musculature without arteriolar sclerosis, the fault may be entirely on the venous side.

#### CASE REPORTS

*Group A. Cases illustrating association of intestinal vascular sclerosis and arteriosclerosis elsewhere in the body.*

CASE 1.—Hospital No. 61437. Pathology No. 8675. Male, aged 45 years, was admitted on Dec. 5, 1935, with a clinical diagnosis of arteriosclerosis, coronary sclerosis, and coronary thrombosis. The blood pressure was 100 mm. systolic and 80 mm. diastolic. A complete history was unobtainable. Death occurred on the same day. At necropsy the relevant findings were an old thrombosis of the left coronary artery, a recent thrombosis of the right coronary artery, with areas of myomalacia. The entire aorta was the seat of

large atheromatous patches and ulceration extending into the common and external iliac branches. The intramural mesenteric arborizations revealed marked tortuosity and thickening. There was marked coronary sclerosis.

CASE 2.—Hospital No. 59727. Pathology No. 9048. Male, aged 60 years, was admitted on Feb. 13, 1936, and died within a few hours. During a previous admission (Oct., 1935) clinical and electrocardiographic examinations (Dr. Capp) revealed coronary sclerosis with myocardial fibrosis. At necropsy, mural thrombi were present at the apex of the heart and in the right auricle. Both coronary vessels were calcified, and a fresh thrombus was present in the right circumflex branch. An old thrombus occluded the left descending coronary artery 2.5 cm. from its origin. The aorta and other large vessels were markedly sclerotic. There was marked tortuosity and thickening of the intramural vascular arborizations with straightening and obliteration of many terminal branches. There was advanced arteriosclerotic renal disease.

CASE 3.—Hospital No. 74062. Pathology No. 11465. Female, aged 77 years, was admitted on Feb. 8, 1937. The patient was known to have had a mild form of diabetes for fifteen years. Some redness, swelling, and tenderness were present on the little toe of the right foot. There was marked peripheral vascular sclerosis. Oscillometric readings at the ankle and above the popliteal space revealed definite occlusive vascular disease. (Doctors Saland and Klein.) Roentgenographic examination (Dr. Snow) showed marked calcification of the vessels of the extremities. Diabetic gangrene of the right foot and lower third of the leg ensued, and the patient died following mid-thigh amputation on March 22, 1937. At necropsy marked atherosclerosis of the aorta with many calcific plaques and ulceration were noted. The aortic and mitral valve flaps were calcified. The larger arterial branches were similarly involved, particularly the coronary and basilar arteries. Typical arteriosclerotic renal disease was present. The intramural mesenteric branches revealed advanced sclerosis, with tortuous, thickened, and partly obliterated straight vessels.

*Group B. Cases with intestinal vascular sclerosis, generalized arteriosclerosis, and marked intestinal clinical manifestations.*

CASE 4.—Hospital No. 74413. Pathology No. 11501. Female, aged 56 years, was admitted on Feb. 19, 1937. The chief complaints were progressive constipation, epigastric discomfort, and belching. The patient was known to have had hypertension for two years. Upon admission the blood pressure was 260 mm. systolic and 135 mm. diastolic. The heart was considerably enlarged. Examination of the fundi revealed dilated veins and thin pale arteries, which were highly refractile and sclerotic. Scattered about the posterior aspect were fine yellowish exudates and a few scattered superficial hemorrhages (Dr. Goodfriend). On Feb. 23 roentgenographic examination revealed calcification of the arch of the aorta. There was loss of substance in the tufts of the index, middle, and little fingers on both sides, indicating circulatory deficiency (Dr. Snow). Oscillometric studies (Doctors Saland and Klein) revealed diminished oscillation in the vessels of both ankles, wrists, and right arm. Thermal tests indicated complete absence of reflex vasodilatation in both extremities. These findings pointed to organic obstructive arterial disease. The patient gradually developed uremia with marked nitrogenous retention and died on March 25, 1937. The relevant necropsy findings were a marked ulcerative atherosclerosis of the entire aorta and small granular arteriosclerotic kidneys. The intramural intestinal arterial arborizations were rigid, thickened, and corkscrew-shaped, with partly or completely obliterated straight terminal branches.

The following brief case reports have been selected from a group of 15 to illustrate the possibilities of accurate diagnosis of intestinal vascular sclerosis by sigmoidoscopy and the value of oxygen as a therapeutic agent.

CASE 5.—A. K., female, aged 55 years, complained of recurring attacks of abdominal cramps, constipation, distention, and a sense of epigastric pressure accompanied by labored breathing, particularly at night. Occasionally there was severe precordial pain radiating to both shoulders. Prior to the onset of these symptoms the blood pressure had been 190

mm. systolic. At the time of consultation it was 150 mm. systolic and 90 mm. diastolic. Cultural, serologic, and parasitologic studies were negative. Sigmoidoscopic and ophthalmoscopic examinations revealed marked sclerosis of the intestinal and retinal vessels. A diagnosis of coronary sclerosis and intestinal vascular sclerosis was made, both being part of a more generalized arteriosclerosis. Considerable relief of abdominal symptoms followed aspiration of the colonic gases and the use of intestinal oxygenation. Several weeks later, after being comparatively free of symptoms, there occurred an acute episode of abdominal pain and an emergency exploratory laparotomy was done at Mt. Sinai Hospital for what appeared to be an acute intestinal obstruction. No obstruction was found, though an omental adhesion in the region of the splenic flexure was present. The striking finding, however, was "a considerable degree of sclerosis in those major abdominal arteries which were seen or felt." 15

CASE 6.—F. K. G., female, aged 64 years, had recurring attacks of abdominal cramps accompanied by nausea for which she had undergone a cholecystectomy eighteen months previous, but without relief. Physical examination revealed a nervous, apprehensive woman, who exhibited evidence of cerebral arteriosclerosis and mental deterioration associated with senility. There was advanced intestinal and retinal vascular sclerosis. The blood pressure was 140 mm. systolic and 100 mm. diastolic. A diagnosis was made of mesenteric vascular sclerosis with relative anoxemia and intestinal spasm. Temporary abatement of symptoms occurred with intestinal oxygenation, but the mental condition of the patient rendered continued therapy difficult. Roentgenographic studies revealed the presence of a diaphragmatic hernia with diverticulosis (Dr. A. F. R. Andresen). There appeared to be no evidence, however, that the intestinal symptoms were due to either of these conditions. Subsequent studies were carried out at Mt. Sinai Hospital in an attempt to elicit an allergic factor, because of a history of migraine, but these were without success. Several weeks later the patient suddenly died of cerebral apoplexy. It was quite evident in this case that not only were the intestinal vessels sclerotic, but the cerebral, aortic, and peripheral blood vessels were likewise affected.

CASE 7.—B. A., female, aged 58 years, had abdominal cramps, distention, and constipation for two years, which was relieved by rest and quantitative, rather than qualitative, dietary restrictions. Repeated roentgenographic studies were essentially negative. Generalized arteriosclerosis was present with moderately advanced changes in the retinal and intestinal vessels. The condition was relieved by the use of approximately 200 c.c. of oxygen administered by rectum once daily. The patient has increased the total amount of food ingested but still eats rather sparingly by choice. The follow-up is now approximately two years, the patient having completely abandoned the use of enemas to which she was previously addicted. At the present time she has one normal bowel movement daily.

CASE 8.—P. Z., female, aged 70 years, complained of obstinate constipation for many years. For several years the patient noted blood in the bowel movements due to the presence of hemorrhoids. Epigastric and precordial distress with a sense of fullness after meals were present for three years. These symptoms were relieved by rigid restrictions in the quantity of food or by a smooth, low residue diet. There was increasing anorexia and a weight loss of 30 pounds. The essential physical findings were abdominal distention, evidence of hypovitaminosis, and generalized arteriosclerosis. The sigmoidoscopic appearance of the intramural intestinal vascular branches was typical of advanced arteriosclerosis, and similar findings were noted in the retinal vessels. The possibility of coronary sclerosis was entertained, but there was no evidence of an acute thrombosis. With intestinal oxygenation the symptoms were ameliorated with a surprising rapidity that could not be accounted for by psychic or other factors. The appetite improved, all dietary restrictions being removed, and with it the state of vitamin deficiency disappeared. Enemas and other forms of catharsis were immediately stopped, and the patient has had normal daily bowel movements, with rare exceptions, for the past five months. In this case it was felt that the oxygenation made possible the handling of large quantities of food by

the intestinal tract. That the total bulk was not an important factor is attested by the fact that the patient had been constipated before dietary restrictions were imposed by her physician.

#### THERAPY

The treatment of intestinal vascular sclerosis is based upon a logical consideration of the physiologic and pathologic factors involved. Our present state of knowledge does not point to any agent which can restore to normalcy the narrowed caliber of mesenteric vessels due to the degenerative changes of senescence. Our efforts must be directed toward supportive and corrective measures which will enable a permanently impaired mechanism to handle adequately the limited digestive requirements of old age.



Fig. 3.—Moderate narrowing of the lumen of a mesenteric intramural branch due to subintimal proliferation. Note that elastica is intact.

(a) *General supportive measures.* If signs of myocardial failure or hypertension are present, adequate drug therapy combined with fluid restrictions should be instituted. Supplementary post-prandial rest periods besides the usual rest requirements will prove helpful.

(b) *Dietary limitations.* The caloric requirements of sedentary old age are obviously much less than those of youth or active middle age. Nature appears to be orderly in her plans. With the vascular and other degenerative changes of senescence, there is a lessened tendency to remain physically active, and with it a diminution in the desire for food. Old people become more “picky” in their food habits and are less apt to overeat than was their custom in youth. Violation of this simple rule often brings on abdominal discomfort

so that individuals soon learn their limitations and steadfastly observe them. Simple, easily digested foods, small in total bulk, well masticated and eaten slowly, are indicated. Ordinarily three meals daily will provide sufficient calorie intake without excess bulk. If not, the same quantity can be divided over five meals. No special food restrictions are indicated, except where specific idiosyncrasy exists. If further restrictions must be made, the addition of vitamin concentrates is advisable. One "fasting" day each week will often be found beneficial, and consists in limiting the food to water, broth, milk, and fruit juices. Although lack of bulk favors constipation, adequate adjustment seems to occur particularly with the aid of intestinal oxygenation.



Fig. 4.—Extreme stenosis of lumen of a submucosal vessel. Note marked subintimal proliferation and several breaks in continuity of the elastica.

(e) *Intestinal aspiration.* By intermittent suction through the sigmoidoscope, distention can be relieved. It also appears to serve as a gentle internal massage to the bowel wall and tends to improve the tone of the musculature. This method is only employed at the beginning of therapy and is later completely replaced by intestinal oxygenation. It is essentially an office procedure carried out under direct vision. Relief is prompt and generally lasts for about twenty-four hours.

(d) *Intestinal oxygenation.* Intestinal oxygenation and the rationale of its employment have been described elsewhere.<sup>16</sup> Normally the large bowel is free or almost free of oxygen. It has been shown that following intestinal oxygenation, the gas is absorbed into and through the wall, increasing the oxygen tension within the blood plasma and the peritoneal cavity.<sup>17</sup> Conversely, the inhalation of oxygen increases the tension within the bowel lumen through the indirect hematogenous route. The bowel wall appears to serve as a semipermeable membrane, diffusion of the oxygen taking place either from the bowel lumen to the plasma or in the reverse direction. Man literally breathes through his intestine. The direct instillation of oxygen into the bowel lumen has been found most effective. It appears to improve the muscle tone and the elasticity of the wall. One of the most striking effects is the relief of constipation. Patients habituated to the use of enemas, who perhaps could

not move their bowels otherwise for three or four days, soon have daily formed bowel movements. The same effect is often noted in young patients. We have been able to demonstrate this effect in some patients suffering from constipation over a period of fifteen years without changing any other factor except the withdrawal of all cathartics and enemas. It is hardly conceivable that the presence of 200 c.c. of oxygen in a colon with a capacity of 2 to 3 liters has any direct mechanical effect by distention of the bowel wall, particularly since the gas is given very slowly. Another interesting observation in connection with the use of oxygen is the rapid disappearance of foul odor from the stool.

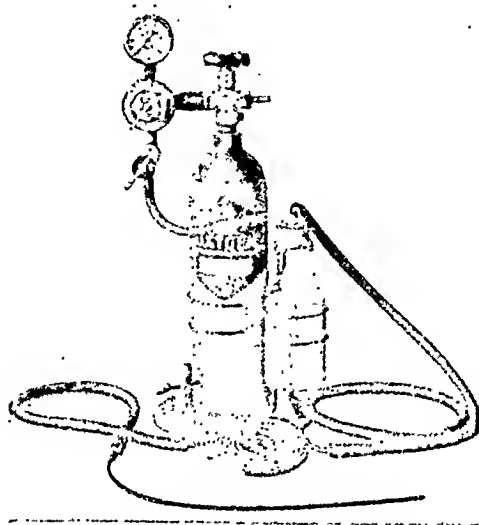


Fig. 5.—Apparatus for intestinal oxygenation, including small warming coil for immersion in basin of hot water or placing upon electric pad. A No. 16 soft rubber catheter is inserted into the rectum.

The oxygen dosage usually employed is approximately 200 c.c., one to three times daily. The apparatus pictured is set up in the bathroom, and the distal 3 or 4 inches of a No. 16 soft rubber catheter lubricated with K-Y jelly is inserted into the rectum. Administered at the rate of 90 bubbles per minute for ten minutes, it will deliver approximately 200 c.c. of oxygen at room temperature. Any similar device may be used. The gas is usually given with the patient in the upright position on the toilet seat, but in debilitated patients it may be administered in the recumbent posture. After proper instruction in the use of intestinal oxygenation, the patient is permitted to continue treatment at home. In some instances the dosage can eventually be cut down after one or two months or even discontinued except for periodic short intervals. During the ten years that intestinal oxygenation has been employed, no untoward result has been reported. In cases where the rectal route is not feasible, the oxygen may be administered by inhalation, though larger amounts are required to relieve intestinal atony.

In closing, it may be stated that no broad generalizations with regard to the therapeutic effects of oxygen are intended. Our experience has been sufficiently large, however, to warrant a rather conservative report so that others may be encouraged to judge of its usefulness.

## SUMMARY

Intestinal vascular sclerosis is essentially a disease of old age. The chief symptoms are distention, constipation, indigestion, and abdominal cramps. Therapy consists of cardiovascular supportive measures, an easily assimilated diet, intestinal aspiration, and intestinal oxygenation.

I wish to acknowledge gratefully the assistance of Dr. I. Rothstein and Dr. W. H. Kastl in reviewing 1,000 necropsy protocols.

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# MONOETHANOLAMINE (MONOLATE)

## AN EXPERIMENT ON ITS ANTIGENIC PROPERTIES

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SINCE the beginning of treatment of varicose veins by injection, there has been a constant search for a more satisfactory sclerosing agent. At the present time sodium morrhuate is the most widely used solution. However, there are numerous reports of allergic reactions to sodium morrhuate, and several deaths, apparently due to allergic reactions, have been reported. These reactions could be expected because sodium morrhuate is made of the sodium salts of the fatty acids of cod-liver oil and is not protein free.

Recently, there has been marketed a solution of monoethanolamine oleate<sup>\*</sup> which closely resembles sodium morrhuate in clinical use and results. Because this solution contains no protein, it was hoped that it would be free of the annoying and occasionally disastrous reactions of sodium morrhuate. A limited clinical trial has shown the material to be very satisfactory. During the period of clinical trial, a death was reported by Shelley,<sup>1</sup> which he ascribed to an allergic reaction to the injection of monolate. Recently, Golden and Heyerdale<sup>2</sup> have reported a case of sensitivity to both monolate and sodium morrhuate in the same individual. Although no other reports of allergic reactions or sensitivity to monolate have appeared in the literature, it was thought that a laboratory test of the antigenic properties of monolate would be desirable. The following experiments were then performed with a comparison of the antigenic action of 5 per cent sodium morrhuate and monolate.

### REPORT OF EXPERIMENTS

Experiment 1. Each of three guinea pigs was injected intraperitoneally with 1 c.c. of 5 per cent sodium morrhuate. Three weeks later each was reinjected with 5 per cent sodium morrhuate. All showed a typical anaphylactic reaction. Two sneezed and became apprehensive; their hair raised, they had a slight convulsion, and recovered. The third had a similar reaction, with a more severe convulsion, and died.

Experiment 2. Each of 12 guinea pigs was injected intraperitoneally with 0.5 c.c. to 1.0 c.c. of monolate, depending on the size of the pig. Three weeks later each was given an intraperitoneal dose of 1.0 to 3.0 c.c. of monolate. There was no reaction in any guinea pig.

Experiment 3. Two cubic centimeters of monolate were injected into the ear vein of a rabbit. Three weeks later 2 c.c. of monolate again were injected intravenously. There was no reaction.

<sup>\*</sup>Monolate.

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Experiment 4. Equal doses of 5 per cent sodium morrhuate and monolate were injected subcutaneously into a rabbit's ears. The size, intensity, and duration of the inflammatory reactions were practically identical.

Experiment 5. (a) Monolate gives a negative reaction to protein by the Greenberg method for total protein. (b) Monolate gives a positive biuret test. This is due to the monoethanolamine, and does not indicate the presence of protein. (c) Micro-Kjeldahl test is also positive. This is due to the presence of nitrogen in monoethanolamine, in the form of an amino group, and again does not indicate the presence of protein.

#### DISCUSSION

The results of this experiment were anticipated on the basis of the chemical compositions of the sodium morrhuate and the monolate. Sodium morrhuate is derived from cod-liver oil, and contains some protein, to which guinea pigs are readily sensitized and show a typical anaphylactic reaction. Monolate contains no protein and apparently will not sensitize guinea pigs. Although it is not possible on the basis of this small number of animals to make a final statement about the antigenic properties of monolate, it is encouraging to find that it is not a sensitizing antigen for any of this group of guinea pigs.

The similar inflammatory reaction produced on a rabbit's ear would reaffirm the clinical impression of a similarity of the sclerosing activity of the 5 per cent sodium morrhuate and monolate. Actually, in practice the monolate seems slightly more potent than sodium morrhuate, and to get a similar reaction, about two-thirds as much monolate as 5 per cent sodium morrhuate can be used.

Of course, no light has been thrown on the problem of drug sensitivity, and anyone who undertakes to give a large number of injections of any drug will sooner or later run across some person who is sensitive to that drug. At the present time no means of predicting such sensitivity has been determined. It has been my custom to give a very small injection of 0.2 c.c. to 0.3 c.c. at the time of the first injection, hoping to measure the patient's reactions to the injection and to the drug. This may be helpful.

#### CONCLUSIONS

No antigenic activity could be demonstrated experimentally in monolate. If it has any antigenic property, it is certainly much less antigenic than 5 per cent sodium morrhuate, which it closely resembles in application and results. Monolate is, therefore, a more satisfactory agent for use in the injection treatment of varicose veins.

I wish to express my thanks to Dr. Paul Micheal for performing the laboratory test and for the use of his laboratory facilities for the animal experimentation.

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## MULTIPLE PRIMARY MALIGNANT LESIONS\*

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MANY reports of multiple primary malignant lesions in the same persons have appeared in the literature since Billroth published the first case in 1869. The first American author to become interested in the problem was Major who, in 1918, reviewed the literature, which was chiefly contributed by German authors. Subsequently Owen (1921), Ophüls (1926), Hanlon (1931), Warren and Gates (1932), Hurt and Broders (1933), Schreiner and Wehr (1934), Bugher (1934), Burke (1936), Anstin (1938), Kirshbaum and Shively (1938), and Stalker and others (1939) have contributed materially to the American literature by publishing large series of cases. In addition, many authors have contributed isolated cases or small series of cases. The summaries of the larger series of cases may be found in Tables I, II, and III where the several series are broken down into small groups because of variations in methods of diagnosis and, particularly in the autopsied and surgical cases, because of the variation in sex incidence and the types of lesions encountered. The Bellevue cases conform in general with those of the quoted autopsy cases.

The Bellevue Hospital material was selected from 6,836 consecutive autopsies performed in the four divisions of the hospital during the past eight years. In 1,044, or 15.2 per cent, of these autopsies malignant lesions were diagnosed, and in 21 of the 1,044 autopsies, or 2.0 per cent, more than one independent malignant lesion was diagnosed. The 21 cases here presented were selected after a review of the autopsy protocols, the microscopic sections, and the clinical histories. The cases are not limited to carcinomas, but include all types of malignant lesions.

The microscopic sections meet the following criteria:

- a. Each lesion possesses generally recognized malignant characteristics.
- b. Each lesion has the histologic pattern commonly associated with malignant lesions arising from the tissue or organ in question.
- c. Each lesion is a distinct entity, metastases having been considered and ruled out in so far as possible in each instance. These criteria closely follow those established by Warren and Gates, and, like theirs, exclude an additional criterion, namely, that each tumor must produce its own metastases, which was advocated by Billroth but which has since been generally discarded.

The clinical histories were studied with a view to establishing the relationship of the duration of the individual lesions and collecting data relative to the familial tendency to malignant diseases. Investigation along both these

\*From the Department of Pathology, Bellevue Hospital, New York.  
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TABLE I  
AUTOPSY SERIES  
(All Diagnoses Confirmed by Histologic Study)

| AUTHOR               | MALIGNANT CASES | MULTIPLE MALIGNANT CASES | PER CENT | AVERAGE AGE | RATIO M:F |
|----------------------|-----------------|--------------------------|----------|-------------|-----------|
| Ophüls               | 512             | 15                       | 2.9      | --          | --        |
| Hanlon               | 950             | 18                       | 1.9      | 62.6        | 13:5      |
| Warren and Gates     | 1,078           | 10                       | 3.7      | 61.8        | 15:25     |
| Bugher               | 983             | 30                       | 3.1      | 61.0        | 24:6      |
| Burke                | 583             | 46                       | 7.8      | --          | 35:11     |
| Austin               | 887             | 24                       | 2.7      | --          | --        |
| Kirsbaum and Shively | 1,411           | 25                       | 1.77     | 63.3        | 17:8      |
| Tullis               | 1,014           | 21                       | 2.0      | 58.4        | 17:4      |

\*This total includes 11 patients with Hodgkin's disease and 23 patients with acute and chronic leucemia. Seven patients with glioma listed elsewhere are not included in this total.

TABLE II  
SURGICAL SERIES  
(All Diagnoses Confirmed by Histologic Study)

| AUTHOR                 | MALIGNANT CASES    | MULTIPLE MALIGNANT CASES | PER CENT | AVERAGE AGE | RATIO M:F |
|------------------------|--------------------|--------------------------|----------|-------------|-----------|
| Hurt and Broders       | 2,124              | 71                       | 3.3      | 50.4        | 32:39     |
| Stalker and co-workers | 2,500<br>(approx.) | 113                      | 4.52     | 59.7        | 50:63     |

TABLE III  
CLINICAL SERIES  
(CLINICAL CASES "OBSERVED")  
(All Diagnoses Not Confirmed by Histologic Study)

| AUTHOR             | MALIGNANT CASES | MULTIPLE MALIGNANT CASES | PER CENT | AVERAGE AGE | RATIO M:F |
|--------------------|-----------------|--------------------------|----------|-------------|-----------|
| Owen               | 3,000           | 143                      | 4.7      | --          | --        |
| Ward               | 1,773           | 94                       | 5.3      | --          | 55:39     |
| Schreiner and Wehr | 11,212          | 307                      | 2.7      | --          | --        |

lines was disappointing. Frequently, unless one of the lesions was on the body surface, ante-mortem diagnosis of the second lesion was not made. As for the familial tendency, in only 3 cases (Nos. 1, 17, 19), or 14 per cent, was there a positive family history of cancer. This is slightly below the figures of 15 to 30 per cent usually reported. However, in one case the causes of death of the patient's family were unknown to him, and in 2 others this information was not obtained before the patients' death. If these 3 cases are subtracted from the total, the percentage of positives becomes 16.6. Even this is believed to be an understatement, since the typical Bellevue patient is drawn from a large city with a large immigrant population. Often he has an imperfect knowledge of English; more often he has lost contact with his family in the fatherland.

Realizing that errors of omission are as unfortunate as errors of commission, a conscious effort to avoid both was made. It was felt that lesions with a known tendency to multicentric origin, such as multiple parenchymal cell carcinomas

of the liver, and lesions of possible malignant origin, which are not yet universally accepted as such, including the leucemias and Hodgkin's disease, should be excluded from this series. Also two cases of bilateral hypernephromas of identical histologic pattern, where it was impossible to rule out the possibility of one being a metastasis from the other, and one case of bilateral carcinomas of the ovaries, where similar conditions prevailed, were not included. However, one case of bilateral carcinomas of the female breasts was included because the one had been surgically removed four years before the appearance of the other, and the two were of different histologic pattern.

TABLE IV

| NO. | SEX | AGE | TYPE LESION                | ORGAN        | ME-TAS-TASIS | TYPE LESION                | ORGAN            | ME-TAS-TASIS |
|-----|-----|-----|----------------------------|--------------|--------------|----------------------------|------------------|--------------|
| 1   | F   | 58  | Perithelial sarcoma        | Liver        | 0            | Adenocarcinoma             | Uterus           | 0            |
| 2   | M   | 44  | Bronchiogenic carcinoma    | Lung         | x            | Squamous-cell carcinoma    | Scrotum          | 0            |
| 3   | F   | 73  | Parenchymal-cell carcinoma | Liver        | 0            | Fibrosarcoma               | Pancreas         | x            |
| 4   | M   | 68  | Squamous-cell epithelioma  | Esophagus    | 0            | Adenocarcinoma             | Prostate         | 0            |
| 5   | M   | 34  | Squamous-cell carcinoma    | Bile ducts   | 0            | Spindle-cell sarcoma       | Stomach          | x            |
|     |     |     |                            |              |              | Adenocarcinoma             | Stomach          | x            |
| 6   | M   | 56  | Adenocarcinoma             | Colon        | x            | Adenocarcinoma             | Prostate         | 0            |
| 7   | F   | 33  | Duct-cell carcinoma        | Right breast | 0            | Colloid carcinoma          | Left breast      | x            |
| 8   | F   | 60  | Adenocarcinoma             | Kidney       | x            | Basal-cell epithelioma     | Cervix           | 0            |
| 9   | M   | 40  | Colloid adenocarcinoma     | Rectum       | x            | Papillary adenocarcinoma   | Transverse colon | x            |
| 10  | M   | 66  | Bronchiogenic carcinoma    | Lung         | x            | Adenocarcinoma             | Prostate         | 0            |
| 11  | F   | 58  | Adenocarcinoma             | Rectum       | x            | Papillary adenocarcinoma   | Rectum           | x            |
| 12  | M   | 76  | Adenocarcinoma             | Prostate     | 0            | Adenocarcinoma             | Pancreas         | 0            |
| 13  | M   | 51  | Adenocarcinoma             | Stomach      | x            | Parenchymal-cell carcinoma | Liver            | x            |
| 14  | M   | 54  | Bronchiogenic carcinoma    | Lung         | x            | Squamous-cell epithelioma  | Tongue           | 0            |
| 15  | M   | 63  | Adenocarcinoma             | Rectum       | 0            | Basal-cell epithelioma     | Temple           | 0            |
| 16  | M   | 50  | Parenchymal-cell carcinoma | Liver        | 0            | Adenocarcinoma             | Stomach          | x            |
| 17  | M   | 58  | Hypernephroma              | Kidney       | x            | Adenocarcinoma             | Pancreas         | 0            |
| 18  | M   | 65  | Adenocarcinoma             | Stomach      | 0            | Adenocarcinoma             | Stomach          | 0            |
| 19  | M   | 75  | Adenocarcinoma             | Stomach      | 0            | Squamous-cell epithelioma  | Esophagus        | 0            |
| 20  | M   | 70  | Adenocarcinoma             | Stomach      | 0            | Adenocarcinoma             | Ascending colon  | 0            |
| 21  | M   | 74  | Adenocarcinoma             | Stomach      | 0            | Adenocarcinoma of polyp    | Stomach          | 0            |
|     |     |     |                            |              |              | Adenocarcinoma of polyp    | Stomach          | 0            |

The cases are listed as a group in Table IV. There are 21 cases, representing 44 lesions, of which 3 are sarcomas and 41 carcinomas. Tables V, VI, VII, and VIII show the organs or organ systems most frequently involved.

The gastrointestinal system is most frequently involved. Twelve cases (57 per cent), representing 20 lesions, are listed under this category. Eight of these 12 cases show 11 lesions in the stomach. Only 2 lesions involve the skin pri-

marily, although 6 lesions arise from stratified squamous epithelium. This distribution is in accord with that in other autopsy series, but is in complete disagreement with the distribution in the surgical and clinically observed cases. In the surgical and clinical series stress is laid on the predominance of skin neoplasms among multiple malignant lesions. Fifty-eight of the 152 lesions among 71 surgical cases reported by Hurt and Broders originated in the skin. Thirty-five of the 113 cases reported by Stalker and associates involved only the skin. Ward, working in England, who reported only rodent ulcers, states that 5.3 per cent are multiple. Since these lesions are by virtue of their location discovered readily and early, they are frequently successfully treated before the patients come to autopsy. If there is a change of hospitals in the interim, the foreign clinical records may be lost to the pathologist.

TABLE V

CASES IN WHICH AT LEAST ONE OF THE LESIONS IS PRIMARY IN THE GASTROINTESTINAL TRACT

| CASE NO. | SEX | AGE | LESION                 | ORGAN   | LESION                     | ORGAN            |
|----------|-----|-----|------------------------|---------|----------------------------|------------------|
| 4        | M   | 68  | Spindle-cell sarcoma   | Stomach | Squamous-cell epithelioma  | Esophagus        |
| 5        | M   | 34  | Adenocarcinoma         | Stomach | Adenocarcinoma             | Prostate         |
| 13       | M   | 51  | Adenocarcinoma         | Stomach | Squamous-cell carcinoma    | Bile ducts       |
| 16       | M   | 50  | Adenocarcinoma         | Stomach | Parenchymal-cell carcinoma | Liver            |
| 18       | M   | 65  | Adenocarcinoma         | Stomach | Parenchymal-cell carcinoma | Liver            |
| 19       | M   | 72  | Adenocarcinoma         | Stomach | Adenocarcinoma             | Stomach          |
| 20       | M   | 70  | Adenocarcinoma         | Stomach | Squamous-cell epithelioma  | Esophagus        |
| 21       | M   | 74  | Adenocarcinoma         | Stomach | Adenocarcinoma             | Ascending colon  |
| 6        | M   | 56  | Adenocarcinoma         | Stomach | Adenocarcinoma of polyp    | Stomach          |
| 9        | M   | 40  | Colloid adenocarcinoma | Stomach | Adenocarcinoma of polyp    | Stomach          |
| 11       | M   | 58  | Adenocarcinoma         | Colon   | Adenocarcinoma             | Prostate         |
| 15       | M   | 63  | Adenocarcinoma         | Rectum  | Papillary adenocarcinoma   | Transverse colon |
|          |     |     |                        | Rectum  | Papillary adenocarcinoma   | Rectum           |
|          |     |     |                        | Rectum  | Basal-cell epithelioma     | Temple           |

The liver is involved in 5 cases, the prostate in 4, the pancreas in 3, the lung in 3, the uterus in 2, and the kidney in 2.

Two cases have 3 primary malignant lesions. Case 4 shows involvement of the prostate (adenocarcinoma), the middle third of the esophagus (squamous-cell epithelioma), and the stomach (spindle-cell sarcoma). Case 21 shows involvement of two distinct polypi in the fundus of the stomach (adenocarcinomas) and an infiltrating lesion in the pylorus (adenocarcinoma).

In 14 cases there are metastases from at least one of the lesions, and in 3 cases from both of the lesions. In 7 cases no metastases could be found.

Seventeen patients were males and 4 were females, a ratio of 4.2:1. Except in one instance (Warren and Gates), the published autopsy series show a comparable ratio of males to females. In the surgical series, however, the ratio

is slightly in favor of the females. The variation may be explained in part by the fact that while women are willing to submit to operation, it is more difficult to obtain permission for post-mortem examination of the female body.

TABLE VI

CASES IN WHICH ONE OF THE LESIONS ARISES FROM STRATIFIED SQUAMOUS EPITHELIUM

| CASE NO. | SEX | AGE | LESION                    | ORGAN     | LESION                  | ORGAN    |
|----------|-----|-----|---------------------------|-----------|-------------------------|----------|
| 2        | M   | 44  | Squamous-cell epithelioma | Scrotum   | Bronchiogenic carcinoma | Lung     |
| 4        | M   | 68  | Squamous-cell epithelioma | Esophagus | Spindle-cell sarcoma    | Stomach  |
|          |     |     |                           |           | Adenocarcinoma          | Prostate |
| 8        | F   | 60  | Basal-cell epithelioma    | Cervix    | Adenocarcinoma          | Kidney   |
| 14       | M   | 54  | Squamous-cell epithelioma | Tongue    | Bronchiogenic carcinoma | Lung     |
| 15       | M   | 63  | Basal-cell epithelioma    | Temple    | Adenocarcinoma          | Rectum   |
| 19       | M   | 72  | Squamous-cell epithelioma | Esophagus | Adenocarcinoma          | Stomach  |

TABLE VII

CASES IN WHICH ONE OF THE LESIONS IS PRIMARY IN THE LIVER OR BILE DUCTS

| CASE NO. | SEX | AGE | LESION                     | ORGAN      | LESION         | ORGAN    |
|----------|-----|-----|----------------------------|------------|----------------|----------|
| 1        | F   | 58  | Perithelial sarcoma        | Liver      | Adenocarcinoma | Uterus   |
| 3        | F   | 73  | Parenchymal-cell carcinoma | Liver      | Fibrosarcoma   | Pancreas |
| 5        | M   | 34  | Squamous-cell carcinoma    | Bile ducts | Adenocarcinoma | Stomach  |
| 13       | M   | 51  | Parenchymal-cell carcinoma | Liver      | Adenocarcinoma | Stomach  |
| 16       | M   | 50  | Parenchymal-cell carcinoma | Liver      | Adenocarcinoma | Stomach  |

TABLE VIII

CASES IN WHICH ONE OF THE LESIONS IS PRIMARY IN THE PROSTATE

| CASE NO. | SEX | AGE | LESION         | ORGAN    | LESION                    | ORGAN            |
|----------|-----|-----|----------------|----------|---------------------------|------------------|
| 4        | M   | 68  | Adenocarcinoma | Prostate | Squamous-cell epithelioma | Esophagus        |
|          |     |     |                |          | Spindle-cell sarcoma      | Stomach          |
| 6        | M   | 56  | Adenocarcinoma | Prostate | Adenocarcinoma            | Transverse colon |
| 10       | M   | 66  | Adenocarcinoma | Prostate | Bronchiogenic carcinoma   | Lung             |
| 12       | M   | 76  | Adenocarcinoma | Prostate | Adenocarcinoma            | Pancreas         |

The average age in this series is 58.4 years. The ages vary from 33 to 75 years, the ages being those at time of death. This average conforms with other published reports. It is slightly above the generally accepted age incidence for single malignant lesions, thereby lending credence to the clinical rule that those tumors which appear late in life are likely to be less rapidly lethal than those which appear early. Being less rapidly lethal, they do not cause the death of the host before a second lesion has time to develop.

COMMENT

The amassed data from several large series of autopsies from American institutions reveal that in 7,448 cases with malignant lesions there are 219 cases (2.8 per cent) with multiple malignant lesions. Kirshbann and Shively, on the basis of their individual data, conclude "individuals may be endowed with a congenital, or acquired, predisposition toward tumor formation." Warren and Gates, and Austin, conclude that the incidence of multiple malignant lesions is

higher than can be explained on the basis of chance alone. A mathematical formula for computing the expected incidence of multiple malignant tumors when compared with the actual facts leads Bugher to conclude that the incidence is "equal to or greater than may be expected from chance alone." Although the percentage of multiples in the Bellevue series is somewhat below that of the total published series combined, the facts seem to warrant concurrence with those whose opinion it is that multiple malignant lesions occur more frequently than can be explained by chance alone. It seems fair to predict that as the life expectancy of patients with cancer is increased due to refinements in diagnosis and treatment, the incidence of multiple malignant tumors will increase and lend more convincing evidence in favor of the foregoing conclusion.

In reviewing the large number of autopsies necessary for the preparation of a paper of this nature, the frequency with which one encounters benign tumors in association with malignant tumors is striking. Studied in connection with family history, the problem is intriguing. It brings up the question of the interrelationship of hereditary predisposition for tumor formation and reaction to environment, and how that interrelationship may affect the health and happiness of the individual. The answer to the riddle is not yet apparent, but the possibility of theory becoming fact should interest the clinician now. With this information in mind he may help his patient and himself in two ways: first, in realizing that his diagnosis is not complete until he has ruled out the possibility of a second tumor once one is found; and, second, in realizing, as Stalker and co-workers have emphasized, the importance of free discussion and investigation of the other members of the family of a cancer patient for the possible presence or development of malignant disease.

#### SUMMARY

1. Twenty-one autopsies with multiple malignant lesions occurring in 1,044 autopsies with malignant lesions are reported. This is an incidence of 2.0 per cent.
2. There were 17 male patients and 4 females, with a combined average age incidence of 58.4 years.
3. Multiple malignant lesions are believed to occur more frequently than can be explained on a basis of chance alone.
4. The clinician is urged to rule out the possibility of a second tumor once one has been diagnosed.
5. The clinician is urged to discuss freely with the family of his patients with cancer the possible familial tendency of the disease.

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## CONGENITAL ABSENCE OF THE GALL BLADDER AND CYSTIC DUCT\*

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### REPORT OF A CASE

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CONGENITAL anomalies of the gall bladder and the biliary ducts have been reported with increasing frequency in the past two decades, but they still remain relatively rare conditions. The importance of these structures in medicine and surgery warrants the description of these anomalies, which may at times assume major significance in diagnosis and treatment.

### REPORT OF A CASE

S. L. (10922-1939), a 76-year-old white male, was admitted to the Metropolitan Hospital on Oct. 17, 1939, with complaints referable to the urinary system. He had received treatment for a mild diabetic condition, but had otherwise been in good health up to the present illness. The family history was irrelevant. There were no gastrointestinal symptoms, except for anorexia and constipation which had developed concomitantly with his urinary complaints.

After physical examination and laboratory work-up, the diagnosis was made of: (1) Fibrosis of prostate with contraction of the bladder neck. (2) Diabetes mellitus. (3) Arteriosclerotic heart disease—compensated. First-stage prostatectomy was performed on Nov. 8, 1939. The patient never rallied following the operation, but went steadily downhill despite all therapeutic measures. He developed an ascending urinary infection, and died Jan. 12, 1940.

During the course of the autopsy it was noted that the gall bladder and cystic duct could not be located. Acquainted with the rarity of this anomaly, we centered our attention on this focus. Careful examination of the skin of the anterior abdominal wall and of the underlying peritoneum did not reveal any scars. The site usually occupied by the gall bladder was free of adhesions or fibrous tissue, which may sometimes conceal an atrophic or shrunken gall

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bladder. The extrahepatic biliary ducts were carefully dissected and their continuity was traced into the duodenum. There were neither signs nor vestige of a cystic duct.

The liver weighed 1,430 Gm. and appeared normal as to shape and relations to the adjacent viscera. The normal markings of the gall bladder fossa were effaced. Glisson's capsule was thickened, and the surface of the liver was finely granular. The organ was firm and cut with resistance. The cut edges were sharp and distinct. The intrahepatic biliary ducts were moderately dilated and filled with viscid green bile mixed with granular debris. The right and left hepatic ducts issued from the transverse fissure, and immediately united to form a dilated common hepatic duct, which was 8 cm. long and had an average diameter of 1 cm. This duct was filled with viscid green bile and showed a small bulbous dilatation 0.5 cm. proximal to its entrance into the duodenum. The duodenal papilla appeared normal. Multiple sections of the liver failed to reveal any trace of an intrahepatic gall bladder. Careful examination of the common hepatic duct did not show any evidence of a cystic duct. The cystic artery was absent.

Microscopically, the capsule was irregularly thickened, and there was extensive subcapsular lymphocytic infiltration. The lobules were irregular in shape, but the relation of the central vein to the lobule was preserved. The perilobular connective tissue was increased and was infiltrated with lymphocytes, plasma cells, and occasional polymorphonuclear leucocytes. There was a proliferation of biliary canaliculi. The larger ducts were dilated and filled with plugs of bile. The hepatic parenchyma, for the most part, appeared normal. However, some cells, especially those at the periphery of the lobules, showed degenerative phenomena and some contained bile pigment. The blood vessels showed thickened walls and were engorged with blood. Sections of the capsule at the site usually occupied by the gall bladder failed to show any evidence of that structure.

The pancreas weighed 78 Gm. and was somewhat indurated. Microscopically, there was a slight increase in the interstitial connective tissue. The islets were abundant and appeared well preserved.

#### DISCUSSION

The significance of anatomic abnormalities of the gall bladder and biliary passages becomes increasingly important as our knowledge of this system progresses. It has been estimated that in at least 10 per cent of all autopsies, some anomaly of the gall bladder, bile ducts, or adjacent blood vessels can be demonstrated.

Agensis of the gall bladder and cystic duct has been described as an independent finding in about 40 cases. More commonly, this anomaly is found associated with other abnormalities of the hepatobiliary system. A total of about 60 cases have been described where absence of the gall bladder and cystic duct are accompanied by other structural defects of this system.

In 1928 Bower,<sup>1</sup> in an extensive review of the anomalies of this system, listed absence of the gall bladder and cystic duct as the fourth most rare anomaly of the hepatobiliary system. He cited 31 case reports of congenital absence of the gall bladder and cystic duct occurring independent of other

anomalies. In that same year, Alglave<sup>2</sup> added another case to this series. Since then cases have been reported, among others, by Danzis,<sup>3</sup> Tallmadge,<sup>4</sup> and Robertson and associates.<sup>5</sup> Knox<sup>6</sup> has reported two cases in a series of 2,000 autopsies. Mentzer<sup>7</sup> and Nagel have seen the condition once each in a series of 1,600 autopsies. Emmert<sup>8</sup> in 1931 reported the first case he had seen in twenty years of experience. Mackinnell<sup>9</sup> in 1930 reported the first case encountered by Schaeffer in 3,000 dissections. This case represents the first instance encountered at the Metropolitan Hospital in a series of 3,660 consecutive autopsies.

If the gall bladder is not found at autopsy or at operation, four possibilities must be borne in mind: (1) The gall bladder has been previously removed. (2) The gall bladder, due to repeated attacks of cholecystitis, has become atrophic or is buried in adhesions. (3) The gall bladder is intrahepatic or is otherwise abnormally located. (4) The gall bladder is congenitally absent.

In the case under discussion there was no history of any attacks of cholecystitis, nor of a cholecystectomy. There were no scars on the anterior abdominal wall nor on the peritoneal surface of the abdominal wall. There were no adhesions nor scar tissue at the gall bladder area. Multiple sections of the liver revealed no evidence of an intrahepatic gall bladder, nor could it be found in any other site. The only possibility that remained was that the gall bladder was congenitally absent.

The gall bladder and cystic duct are normally absent in certain species of animals (horse, mule, elephant). Some species may or may not have it (giraffe). In certain animals (pigeon) a gall bladder is present in embryonic life but disappears in later development.<sup>9</sup> Its absence in man has been regarded by some writers as atavistic.

Congenital anomalies of the hepatobiliary system are of importance to the surgeon and clinician as well as to the pathologist and anatomist. These anomalies may lead to erroneous diagnoses, as when the gall bladder fails to visualize on cholecystography. They may also give rise to technical difficulties in surgical procedures. We recently had the opportunity to see a case where a cholecystogastrostomy was performed for common duct obstruction. The operation was technically successful, but at autopsy we found that the cystic duct ran an independent course up to immediately before the ampulla of Vater. There was not sufficient intraductile pressure to force the accumulated bile from the common duct up the long cystic duct into the gall bladder, and through the stoma between the gall bladder and the stomach. Had this anomaly been recognized at operation, an attempt might have been made to anastomose the widely dilated common hepatic duct to the stomach or duodenum, thereby prolonging the life of the patient.

Dilated bile ducts often, but not constantly, are found associated with absence of the gall bladder. Waring<sup>10</sup> was the first to make the observation that "when the gall bladder and cystic duct are absent, the common duct is usually found to be enlarged a short distance above the entrance into the duodenum, so as to form a fusiform sac, which may serve to a certain extent, as a substitute for the gall bladder in the storage of bile which is secreted between the periods of digestion." Such a localized dilatation was found in this case in an already dilated common hepatic duct. It is interesting to note

that in agreement with Bower's statistics, the deceased showed dilated ducts and had lived to 76 years of age. Bower<sup>1</sup> states the average age of those who died with normal ducts was only 36, as compared with 52 years for those whose hepatic ducts were dilated. This is in accord with the fact that in some species of animals that are not provided with a gall bladder, the common duct dilates to compensate.

#### CONCLUSION

1. Congenital absence of the gall bladder and cystic duct is a relatively rare entity.

2. A case is reported where this condition was found at autopsy, but there were no symptoms clinically to indicate the presence of this anomaly.

3. Anomalies of the hepatobiliary system are of prime importance in the medical sciences.

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# OBSERVATIONS ON CAPILLARY PERMEABILITY AND INFLAMMATION IN THE SKIN OF SENSITIZED RABBITS\*

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TRYPAN blue when given intravenously to normal rabbits localizes and concentrates in areas of inflammation only during a specific interval. This interval is not determined by the presence of edema, hyperemia, or polymorphonuclear leucocytes.<sup>1-3</sup> It has been suggested that this colloidal dye localizes and concentrates in areas of inflammation as a "result of a temporary change which occurs in the epithelial cells, the connective tissue cells, and the endothelial cells of the capillaries. This change in the permeability of these cells results from the effect of xylol on the epithelial cells. The normal metabolism of the epithelial cells is disturbed, as shown by the presence of pyknosis, karyorrhexis, and necrosis. The results of the effects of this abnormal intercellular fluid on the capillary endothelium is, no doubt, a compensatory mechanism, permitting the passage of water and crystalloids at a more rapid rate than normal and also readily allowing the passage through the vessel wall of the cells, colloids, and plasma proteins."<sup>4</sup>

Ramsdell<sup>5</sup> injected trypan blue intravenously to demonstrate the immediate skin reaction in rabbits and guinea pigs made hypersensitive to horse serum. The dye was injected intravenously immediately before 0.01 c.c. of horse serum was given intradermally. The infiltration of dye into these reactive sites, according to Ramsdell, "is at present to be looked upon as no more than a sort of indicator of the presence of an edematous change which is an expression of a toxic action upon the capillary endothelium." In discussing the observations of Ramsdell, Burrows<sup>6</sup> states that "it would be of interest to know the results if these experiments were repeated with controls."

The present paper is first a report of our observations on the localization of trypan blue in the skin of normal and sensitized rabbits following the intradermal injection of horse serum, and secondly, our observations on the effect of trypan blue on the development of the local reaction following an intradermal injection of horse serum in the skin of sensitized rabbits.

## METHODS AND MATERIALS

The skin of the rabbits is carefully shaven twenty-four hours or longer before the horse serum† is injected intradermally. The serum is injected at

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varying intervals before 10 c.c. of a 0.2 per cent solution of trypan blue is given intravenously. The skin is observed for an hour for the appearance of a blue color.

A series of intravenous and intraperitoneal injections of horse serum is given for the production of sensitivity. The serum, obtained by bleeding from the heart, is titrated for precipitins in those rabbits used to study the effect of trypan blue on the skin reactions. The horse serum for intradermal injection is diluted with saline.

#### EXPERIMENTAL

*Localization of Trypan Blue in Areas of Skin Injected With Horse Serum. Normal and Sensitized Rabbits.*—Eight normal rabbits are injected intradermally with 0.2 c.c. of horse serum at the following intervals before 10 c.c. of a 0.2 per cent solution of trypan blue is given intravenously; twenty-four hours, five hours, three hours, one hour, thirty minutes, and immediately. In the normal rabbits only the last two areas, where the serum is injected immediately before the dye is given, become blue within a period of thirty minutes. In a group of six sensitized rabbits the dye localizes and concentrates in the areas of skin injected with the horse serum as long as three hours before the dye is given.

The greatest amount of dye concentrates in the areas injected with the serum the shortest time before the dye is given. A larger area of blue occurs at the site of the intradermal injection in the sensitized rabbits than in the normal rabbits.

There is only a small amount of edema in the skin at the site of the intradermal injections of the serum in the normal rabbits. In contrast to this, there is extensive edema and hyperemia in the skin where the horse serum is injected in the sensitized animals.

These observations show that a similar change occurs in the skin of both the normal and the sensitized rabbit following the intradermal injection of horse serum which permits the localization and concentration of trypan blue. This change persists longer, however, in the sensitized animals than in the normals. The localization and the concentration of this dye are not determined by either the presence or the absence of edema and hyperemia. There are considerable edema and hyperemia in the area where horse serum is injected twenty-four hours previous to the injection of the dye in the sensitized rabbit. The dye fails, however, to concentrate in the area. These observations seem to support the idea previously expressed that trypan blue localizes and concentrates in areas as a result of a change in the tissue cells. This change is a temporary one. The capillary endothelial cells, the tissue cells, and the epithelial cells are all apparently affected.

*Effect of Trypan Blue on the Development of the Local Reaction in the Skin of Sensitized Rabbits.*—It seems of interest in this experiment to study the effect of trypan blue on the development of the local reaction in sensitized animals. The results of the preceding experiments support the opinion previously expressed that trypan blue localizes and concentrates in areas of inflammation as a result of a temporary change in the endothelial cells, the con-

nective tissue cells, and the epithelial cells.<sup>4</sup> Moon<sup>7</sup> thinks that the above cells become sensitized and are injured directly by contact with the specific antigen. The presence of a colloid in these cells might influence their response to a specific antigen.

Five rabbits sensitized to horse serum are used in this experiment. Five days before the intradermal injection of the horse serum, the precipitin titer of the serum from these rabbits varied from 1:320 to 1:5,120. Ten cubic centimeters of a 0.2 per cent solution of trypan blue are injected intravenously into each of three of these rabbits, and into one control thirty hours and again six hours before the intradermal injection of 0.1 c.c. of the following dilutions of horse serum: undiluted, 1:10, 1:20, 1:40, 1:80, and 1:100. The skin is dark blue when the horse serum is injected. There are edema and hyperemia after twenty-four hours in each area of skin injected with the serum diluted as much as  $\frac{1}{40}$  in each of the sensitized rabbits, while only a small reaction is present in the normal rabbits where undiluted horse serum is injected. The skin in the two sensitized rabbits without dye corresponds exactly with the reaction in the three rabbits given the trypan blue.

It may be concluded from this experiment that trypan blue does not affect the reaction of sensitized cells to a specific antigen.

#### DISCUSSION

The observations made in this experiment that trypan blue following an intravenous injection localizes and concentrates only during a specific interval following the intradermal injection of horse serum corresponds to other studies where this dye was found to localize and to concentrate in areas of inflammation produced by the local application of xylol only during a specific interval.<sup>1, 2</sup> The present experiment also shows that the time in which trypan blue localizes and concentrates in an area is not determined by the presence of edema and hyperemia. The localization and the concentration of this colloidal dye in specific areas of skin supports the opinion that "the connective tissue and the vascular system differs as far as their functional state is concerned in sensitized and non-sensitized animals."<sup>8</sup>

There are many technical difficulties that one encounters in an attempt either to saturate or to block and then to study the reaction of specific cells. Cannon and his associates,<sup>9</sup> and Jaffe<sup>10</sup> have discussed these in considering the effect of blocking the reticulo-endothelial system on the formation of antibodies. The occurrence of edema and hyperemia at the site of the injection of the antigen in the sensitized rabbits given trypan blue similar to that occurring in rabbits without this dye certainly supports the opinion that "the storage of the dye does not affect the vital parts of the cells and in the dye-filled cells sufficient cytoplasm is left to carry on its metabolic processes. The cell also is able to take in more foreign material of the same or of another kind."<sup>10</sup>

These observations show that trypan blue localizes and concentrates in areas of skin injected with horse serum in sensitized rabbits during a specific interval. Furthermore, the greatest quantity of dye localizes and concentrates

in the area of skin injected for the shortest interval before the dye is given. These results suggest that immediately following the contact of the antigen with the sensitized cells a change occurs in the cells which permits the entrance of the colloidal dye. This change in the cell is transient. If the cell is completely destroyed, the coagulated cytoplasm may become blue by a diffusion of the colloidal dye from the intercellular fluid. In the rabbits where extensive injury occurs following the intradermal injection of horse serum, trypan blue may be observed to localize at the periphery of the lesion for as long as twenty-four hours. The localization of dye at the periphery of such a lesion must be differentiated from the localization of the dye which occurs throughout an area infiltrated by the horse serum. In the former, the cells are affected by being proximal to severely injured tissue, while in the latter the localization and the concentration of dye apparently results from the effects produced by the contact of the antigen with sensitized cells. It must be remembered that the metabolism of a cell may be disturbed by many different processes.

The Arthus' phenomenon is not influenced by the intravenous injection of ink given before and after sensitization.<sup>10</sup> Although the reaction produced by the intradermal injection of horse serum into a sensitized rabbit may be different from the Arthus' phenomenon, it is of interest to know that trypan blue and ink do not affect the development of either the hypersensitive reaction or the Arthns' phenomenon.

#### SUMMARY

Trypan blue following an intravenous injection localizes and concentrates in areas of the rabbit's skin injected with horse serum if the serum is injected a short time before the dye is given. The time during which this dye localizes and concentrates in the skin is much longer in sensitized rabbits than it is in the skin of normal rabbits.

The localization and the concentration of trypan blue in areas of inflammation produced by horse serum are not determined by the presence of edema and hyperemia. This dye may not concentrate in all areas of edema and hyperemia, as shown by the failure of the dye to concentrate in areas of skin injected with horse serum twenty-four hours previously to the injection of the dye.

The presence of trypan blue in the tissues of a sensitized rabbit apparently does not affect the subsequent reaction that occurs when the antigen is injected intradermally.

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## PERCUSSION AND AUSCULTATORY PERCUSSION WITH A NEW INSTRUMENT\*

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**I**N ASCERTAINING the condition of the lungs, the size of the heart, great vessels, and viscera, no clinical method will ever have the finality of the roentgen ray. However, clinical means of evaluation of these facts, such as percussion and auscultation, are very important in the everyday practice of medicine, and any instrument that might improve our ability to recognize variations from normal justifies its existence. Moreover, competent evidence exists that percussion and its modifications can be fairly exact if done carefully.<sup>1</sup>

Ever since Auenbrugger first published his results obtained from percussion in 1761, there have been attempts at variation of this clinical method.<sup>2</sup> The pleximeter was first invented by Piorry of Paris in 1828, and has been constantly improved since then. During the past three years, after working with several hollow pleximeters in order to magnify the sound conduction, I selected one which gave very satisfactory results. It consists of an instrument called the Fox localizing attachment, whose original purpose was for localizing cardiac murmurs in auscultation of the heart. For its new use it has been renamed the resonant pleximeter. The instrument consists of a localizing rod attached to a resonating chamber or sounding box, and is used in percussion instead of the pleximeter finger. The use of the resonant pleximeter in percussion of the chest, the heart borders and great vessels, gave a more discernible change between resonance, flatness, and dullness, than was possible without this aid.

While experimenting with this instrument, it was attempted to lead off the sound obtained from percussion directly to the ears of the examiner; in other words, to employ auscultatory percussion. It was found at first that the sound reaching the examiner's ears was too loud to be analyzed accurately. A plug of cotton inserted into the instrument where it is attached to the stethoscope tubing, sufficiently muffled the sound so that it can be properly evaluated. Loudness is not a criterion in its use. In the determination of the size of heart, it is preferable to start in the center of the anterior chest wall over the sternum and lightly percuss the pleximeter as it is slowly moved in each direction, until a change in quality of the sound is noted. Care must be taken to

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tap lightly with the ball of the finger, and to keep the strokes of the finger of approximately the same intensity. Care must also be taken that the localizing rod is between the ribs and not on the ribs, because the ribs conduct and emit sound differently than do the soft tissues. In our opinion other organs, such as the liver, spleen, consolidated areas in the chest and pleural effusions can also be percussed with fair accuracy. In the case of the liver, the instrument is placed over the apparent center of the liver anteriorly in the mid-clavicular line, and percussion is employed in each direction until a definite change in the quality of sound is elicited.

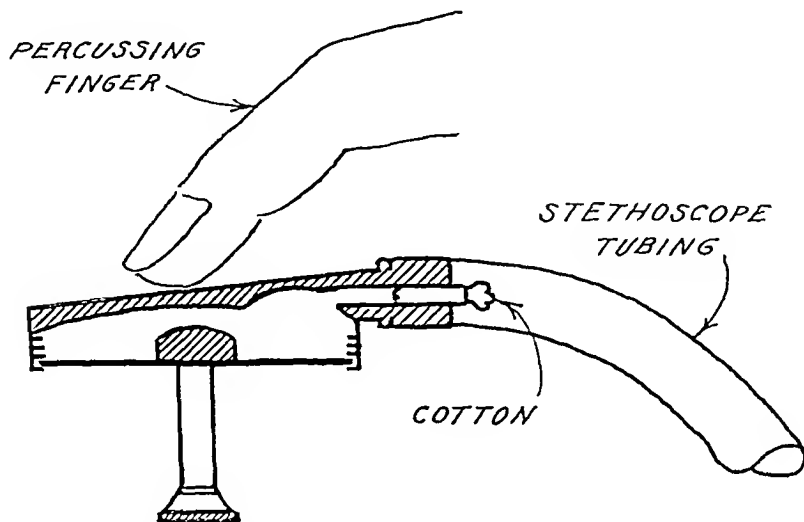


Fig. 1.—The resonant pleximeter for auscultatory percussion.

Certain difficulties inherent in the entire study of percussion must be considered at this point, so that any method may be properly evaluated.<sup>3</sup> It must be understood that the word resonance can mean all things to all men. It cannot be accurately described. Each individual has his own standard for comparison. What is important is that the interpretation based on this standard should be the same in each case. To achieve this, nothing will take the place of practice. The percussion sound is produced by successive layers of the chest; a light stroke will bring into vibration the superficial layers and organs, and a heavy stroke the deeper layers. The pleximeter finger or instrument must be placed fairly snugly against the chest wall so as to damp the vibrations of the chest wall and bring out the vibrations of the tissue that we intend to percuss. Each percussion note consists of a predominant or fundamental tone and overtones. To evaluate the percussion note properly, we have to disregard the overtones. The percussion note also depends on such seemingly insignificant aspects, such as the pedestal on which the subjects rest, e.g., whether the subject stands on a stone floor or rests in bed. In the former case, there is a diminution of overtones; in the latter, an accentuation. It would, therefore, be better to have the subject standing or sitting on a hard object, rather than lying on a soft bed. This is a very important factor in reducing the number of overtones. The fundamental or predominant tone has several character-

istics, such as pitch and resonance. The pitch is determined by the rate of vibration of the organs percussed. If the rate of vibration is high, the pitch is high. The resonance of a body depends on its ability to keep vibrating when struck. If the body stops vibrating after it is struck, it is nonresonant. The pitch and resonance both depend on the tension, thickness, extent, and constitution of the matter of the body that is vibrating. The tension and thickness for example might be different in the same pathologic conditions in different individuals; thus a pneumothorax note may be of high or moderate pitch, depending on the tension in the pleural cavity.<sup>3</sup> It can thus be seen that no instrument can possibly take the place of careful analysis of the percussion note.

#### HISTORICAL

The historical development of auscultatory percussion is interesting, because it shows how widely different the opinions of reliable observers can be as to its validity.<sup>4</sup> The instrument here suggested, we believe, circumvents the difficulties experienced with the older instruments, because it utilizes a direct method of percussion, and an evaluation of this percussed area by auscultation. The following historical sketch will show the difficulties inherent in this system of clinical diagnosis, and will show how some of these have been surmounted.

Auscultatory percussion was first suggested by Laënnec in his *Traité de l'auscultation médiate*,<sup>5</sup> but he did not use it to determine the size of the heart. Cammann and Clark<sup>6</sup> were the first actually to attempt to determine the size of the heart. One end of a rod was placed against the chest wall of the subject and the other in the ear of the examiner. The examiner started percussing a distance away from the organ and gradually approached it. The end point was reached when there was a sudden increase in loudness as the organ was approached. Subsequently, nearly all improvements in auscultatory percussion have been variations of the observations of Cammann and Clark, the later investigators using percussion in concentric circles, either toward or away from a stethoscope placed over the organ. Bianchi<sup>7</sup> in 1884 replaced the stethoscope with the phonendoscope, which had a rod with a small resonating chamber above it, and used scratching of the skin instead of percussion. In the ensuing years attention was centered in modifying the types of percussion used. Very light taps were used by some. Others used friction on the skin with the fingernails, going in concentric circles toward the organ.<sup>8</sup> Still others used friction strokes with the brush, moving away from a stethoscope placed on an organ.<sup>9</sup> Later, automatic hammers were used in conjunction with the stethoscope.

Most investigators placed great stock in their ability to outline the borders of the heart, abdominal masses, liver, and spleen with auscultatory percussion. Pal was able to diagnose two echinococcus cysts rather than one cyst in the liver of a patient.<sup>8</sup> The enthusiasm of these early workers knew no bounds; some even felt able to discern the place of the interventricular septum. Then came the inevitable reaction with the researches of de la Camp<sup>10</sup> and Selrwald.<sup>11</sup> These authors, working on cadavers, showed that by placing the stethoscope at a certain spot and percussing toward it, they were able to shift the area of

the apparent left border of the heart at will. They concluded that certain friction patterns that were assumed to map out the size of the heart were really the result of conduction of the percussed patterns along ribs, sternum, muscle, and bone, and thus could be shifted at will if the stethoscope was moved.

More modern investigators<sup>12, 13</sup> seem to have overcome the objections of the early workers by using some object for direct percussion, and leading the sound thus elicited to the ears of the examiner. Auscultatory percussion with the resonant pleximeter falls into this class, although the instrument used was independently studied and does not resemble the other available instruments in any of its aspects.

#### COMMENT

The advantages of the instrument here discussed are:

1. It utilizes a direct percussion of the underlying structures and thus appears to do away with the disadvantage of the early methods.

2. The instrument can be easily procured at the present time and adapted for use.

#### SUMMARY

The instrument called the resonant pleximeter has been adapted for use in auscultatory percussion.

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Mr. Fleischer, of the Becton-Dickinson Company, cooperated in this study by supplying various types of pleximeters. The instrument used in this study can now be purchased in the market as the Fox localizing attachment.

# CLINICAL CHEMISTRY

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## THE INTRAVENOUS USE OF A SUCROSE-RINGER'S SOLUTION TO PRODUCE MAXIMAL DIURESIS\*

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IN A PREVIOUS communication we reported on the determination of the relative merits of a series of solutions to produce diuresis without danger to the animal or injury to its kidney. The most consistent producer of diuresis which approximated the intravenous intake was 8 per cent sucrose in half strength Ringer's solution. When this was given for periods of six hours to each of eight rabbits at a rate of 100 c.c. per kilogram per hour, the animals all survived, and the average output was 88 per cent of the amount given in the six-hour period. At the time we reported that two of the animals had received multiple six-hour injections without serious difficulty and with a slightly greater diuresis in the later injections. Recently, Anderson and Bethea called attention to the marked changes in the convoluted tubules of the kidneys of persons who shortly before death had received sucrose solutions intravenously. It is undoubtedly true that these changes did not benefit the individual, but it is also true that they probably did him very little harm. After our work on both rabbits and dogs it seems reasonable to assume that the cell of the convoluted tubule can appear very much injured histologically and yet function normally, and can in a short time again return to normal appearance.

Our object in this study was to determine the innocuousness of repeated six-hour intravenous injections of sucrose-Ringer's solution when given at intervals of about a week. For this purpose we used three rabbits of about 2.5 kg., each animal receiving 100 c.c. per kilogram per hour for six hours. One animal received six, the second ten, the third fourteen, injections.

The first animal was killed three days after the sixth injection; the second animal died during the fourth hour of the eleventh injection, death being caused by the accidental injection of air; and the third died a traumatic death six days after its left kidney had been removed. In view of the changes occurring in the urine twenty-four to forty-eight hours after the first injection, two other animals received injections, one a single, and the other two.

In order to determine its value in other species of animals, the same solution was used in dogs in quantities of 50 c.c. per kilogram per hour for six hours.

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Three of the dogs received two injections only. The first dog received two injections on succeeding days; the second received the second injection forty-eight hours after the first; the third received the second injection one week after the first. The fourth dog, weighing 9.0 kg., received twenty-eight injections, twenty-seven before and one injection after removal of its left kidney. The following determinations were made routinely: blood urea, phenolsulfonphthalein output, output during six-hour diuresis, output during twenty-four-hour periods in the interval between injections, and specific gravity. Albumin, casts, erythrocytes, and leucocytes were tested for in the urine. A brief résumé of the experiments will show the trends without too much detail.

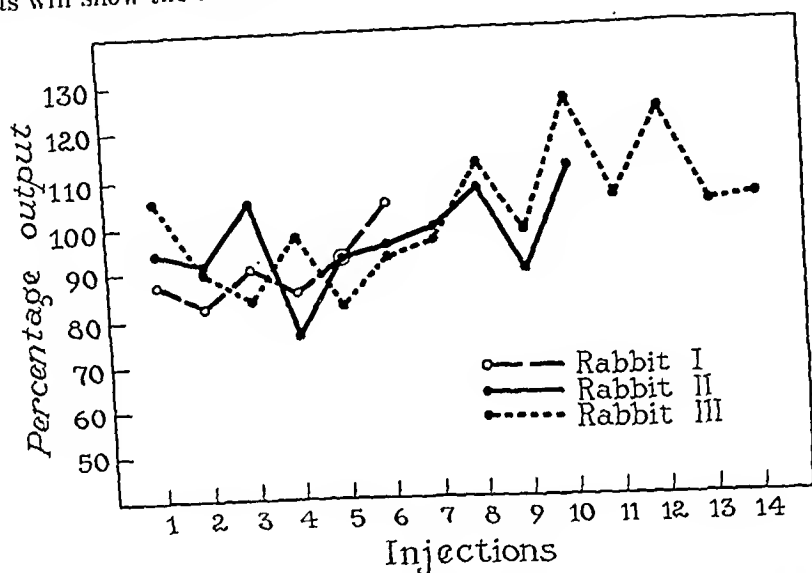


Fig. 1.—Output of urine after intravenous injection of sucrose-Ringer's solution as percentage of the amount injected.

Rabbit 1, weighing 2.3 kg., received six injections of 1,380 c.e. each of sucrose-Ringer's solution. The output varied from 1,150 to 1,440 c.e., or 83 to 104 per cent of the amount injected (Fig. 1); the average output was 90 per cent. The blood urea was 34 mg. per 100 c.e. before starting the injections, and at no time did it rise above 39 mg. The phenolsulfonphthalein output was 67 per cent before the start of injection and varied between 90 and 42 per cent. There was albumin, grade 3, in the urine in the second twelve-hour specimen after the first injection, and a trace on two occasions after that. Otherwise the urine was free of albumin. There were some erythrocytes and leucocytes in the specimen of urine taken forty-eight hours after the first injection; otherwise there were no formed elements. The animal was killed three days after the sixth injection. The findings at necropsy were essentially negative. Grossly, the kidneys were slightly enlarged and lighter in color than normal. Histologically, the convoluted tubules showed marked vacuolar degeneration and swelling of the cells, up to obliteration of the lumina of some of the tubules. The glomeruli looked compressed.

Rabbit 2, weighing 2.5 to 2.8 kg., was given ten injections of 1,620 c.c. each of sucrose-Ringer's solution. The output ranged from 1,235 to 1,810 c.c., or 76 to 112 per cent. Two of the last four injections produced diuresis of more than 100 per cent. The average output was 95.9 per cent of the amount injected. The blood urea ranged from 18 mg. per 100 c.c. to a high of 39 mg. after the fourth injection, with a return to 30 mg. at the time of death during the eleventh injection. The phenolsulfonphthalein output ranged from 60 to 97 per cent. There was albumin, grade 2, in the urine after the first injection only, with many casts after the first and seventh injections, and leucocytes after the first, second, seventh, and eighth injections. Fig. 1 (in the solid line) gives the percentage output of the various injections, showing the relatively better output in the later injections. The animal died and the kidneys were removed immediately and fixed in formalin. No cause of death other than air embolism was found, all the organs showing practically a normal state. The kidneys were not much enlarged.



Fig. 2.—Separation of tubules in the kidney of a rabbit. The kidney was removed during the fourth hour of the eleventh injection of sucrose-Ringer's solution ( $\times 190$ ).

They were brownish red and slightly lighter than the normal kidneys. The capsule was not adherent. The pelvis looked normal. On section the cortex was a little lighter in color than the medulla.

Microscopically, the striking feature was the uniformity of the cortex. All cells of the convoluted tubules were light pink, owing to staining of a finely granular protoplasm small in amount. The collecting tubules were lined by a low epithelium, which stained a uniform red in contrast to the pink convoluted tubules. Most of the convoluted tubules had a good-sized lumen. A few were almost swelled shut. Most of the nuclei were oval; some were pyknotic and shrunken. Some glomeruli seemed to have been distended; in others the outer wall of the capsule was fairly close to the glomerular tufts. In many areas there

was a considerable separation of the tubules as if by fluid (edema). Fig. 2 illustrates this condition of the kidney parenchyma.

Rabbit 3, weighing 2.3 to 2.9 kg., received fourteen injections of 1,560 c.c. each of sucrose-Ringer's solution. The output during the six-hour periods ranged from 1,285 c.c. to 1,890 c.c., an output of from 82 to 121 per cent. The average output in the six-hour period was 101 per cent; in seven injections the output was greater than 100 per cent, and in seven it was less than 100 per cent. The blood urea ranged from 14 to 30 mg. per 100 c.c. The maximal reading was 30 mg. per 100 c.c. after the third injection; the reading before the fourteenth injection was 18 mg. per 100 c.c. The single reading of the phenolsulfonphthalein output taken before the first injection was 67 per cent. All readings after this ranged between 82 and 96 per cent, except that twenty-four hours after the eleventh injection, which was given two days after the tenth injection, the phenolsulfonphthalein output dropped to 44 per cent. Five days later it was again 91 per cent. There was albumin, grade 1, in the urine twenty-four hours after the first injection and albumin, grade 4, after the second, but no albumin in the urine after any of the other injections. There were casts, grade 2, erythrocytes, grade 1, and leucocytes, grade 2, after the second injection, but they were absent after the other injections. Three days after the fourteenth injection the left kidney was removed. Six days later the animal died as a result of an injury sustained at the time of biopsy. Fig. 1 shows the percentage output of rabbit 3.

Tissue was removed from the rabbit three days after the fourteenth injection and again nine days after this injection. The specimens removed three days after the fourteenth injection were two small wedge-shaped pieces taken from the left kidney. The epithelium of the convoluted tubules was about normal in height and had lost its light, granular appearance. The protoplasm again stained a definite red and was collected toward the basement membrane. Strands extended out into the lumen, possibly the remains of the vacuolar degeneration. In some areas the vacuolar degeneration still remained more marked than in others. The glomeruli showed no change, and no mitotic progress was found in the convoluted epithelial cells.

The specimen removed shortly after death on the ninth day after the fourteenth injection showed a normal picture in the right kidney, but considerable change in the left, from which two wedges of tissue had been removed. In the left the convoluted epithelium still showed some vacuolar degeneration, and most of the tubules were filled with a pink, finely granular detritus. The right kidney could be very simply described as the picture of the normal rabbit kidney. The three photomicrographs show well the rapid return to normal of the epithelium after a marked functional change which gives the kidney the peculiar appearance seen in Fig. 2. Fig. 3 shows a condition of the convoluted epithelium after a period of three days, and Fig. 4, an almost complete return to normal after nine days.

In order to check still further the effects of the first and second injections, which seem to give the most marked reaction in the urine, two other rabbits were given injections, one a single, and the other two.

Rabbit 4, weighing 3.3 kg., put out 94 per cent of the 1,980 c.c. of sucrose-Ringer's solution injected in the first six-hour period and 84 per cent in the second. After the first injection the twenty-four-hour urine showed albumin, grade 4+, no casts, and a considerable number of leucocytes. The following twenty-four-hour specimen had a trace of albumin and no leucocytes. The phenolsulfonphthalein output dropped from 93 to 40 per cent in the twenty-four-hour period after the first, and from 87 to 55 per cent in the twenty-four hours after the second injection. The left kidney was removed twenty-four hours after the second injection. There was marked hydropic degeneration. The restitution to normal in the right kidney removed a week later was not nearly as complete as in the experiment in which only one injection was given.



Fig. 3.—Appearance of the convoluted tubules in the kidney of a rabbit three days after the fourteenth injection of sucrose-Ringer's solution ( $\times 145$ ).

Rabbit 5, weighing 2.2 kg., excreted 115 per cent of the 1,320 c.c. of sucrose-Ringer's solution intravenously injected in the six-hour period. In the twenty-four-hour period following the injection there were albumin, grade 4, erythrocytes, grade 1, and leucocytes, grade 1, but twenty-four hours later there was no albumin, erythrocytes, or leucocytes. The phenolsulfonphthalein output dropped from 75 to 49 per cent. The left kidney, removed twenty-four hours after the injection, showed marked hydropic degeneration and raggedness of cells. The right kidney was removed at necropsy one week later and showed practically a normal renal structure.

In order to determine what diuresis smaller quantities of 8 per cent sucrose in half strength Ringer's solution would produce, we gave two animals 50 c.c. of the solution per kilogram per hour for six hours, and two animals 25 c.c. per kilogram per hour.

Rabbit 6, weighing 2.1 kg., received 630 c.c. (50 c.c. per kilogram hour) and excreted 498 c.c. (38.5 c.c. per kilogram hour), or 79 per cent, in the six-hour



period. Rabbit 7, weighing 2.1 kg., received 630 c.c. (50 c.c. per kilogram hour) and excreted 446 c.c. (35.5 c.c. per kilogram hour), or 71 per cent, in the six-hour period. The average for the two rabbits was 75 per cent. Rabbit 6 was killed after twenty-four hours, and rabbit 7 after forty-eight hours. The changes were about as great as were those at a similar interval after injections of 100 c.c. per kilogram per hour for six hours. There was great vacuolar degeneration, irregularity of outline of the cells, and swelling of the cells to obliteration of the lumen of the tubules.

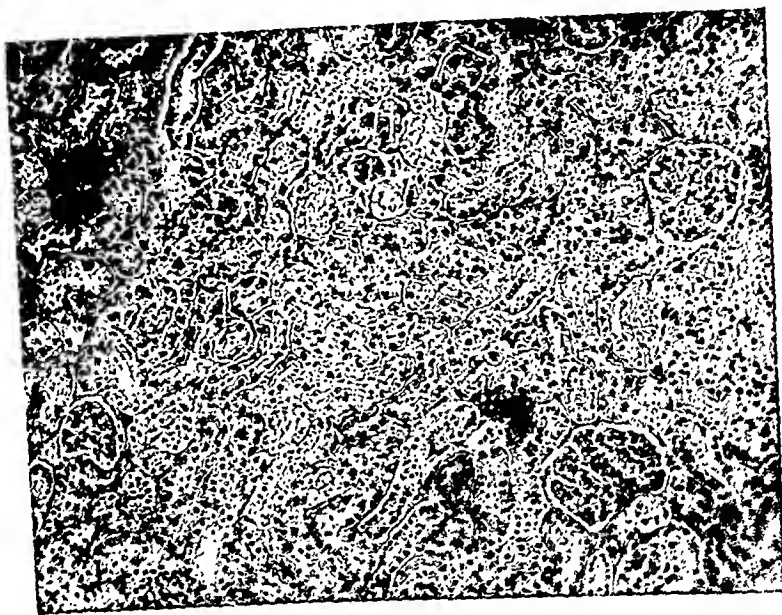


Fig. 4.—Return to normal of the convoluted tubules in the kidney of a rabbit nine days after the fourteenth injection of sucrose-Ringer's solution ( $\times 125$ ).

The two animals that received only 25 c.c. per kilogram per hour for six hours excreted, one 31 per cent (8 c.c. per kilogram hour), and the other 50 per cent (12.5 c.c. per kilogram hour) of the amount injected, an average of 40 per cent, indicating the necessity of greater amounts of sucrose to produce diuresis. The histologic changes twenty-four and forty-eight hours after diuresis showed marked hydropic degeneration in the convoluted tubules. This was greater in the kidney removed after twenty-four hours than in the kidney removed after forty-eight hours.

Inasmuch as the rabbits that received 100 c.c. per kilogram per hour of the 8 per cent sucrose solution all survived, we thought it would be interesting to see the limit of the excretory capabilities of the rabbits' kidneys. Therefore, one animal was given 125 c.c. per kilogram per hour and another 150 c.c. per kilogram per hour. The animal receiving 125 c.c. per kilogram per hour put out 68 per cent (85 c.c. per kilogram hour) of the 2,172 c.c. injected, and showed no ill effect from the injection. The animal receiving 150 c.c. died forty-eight hours after the injection, having put out 88 per cent (132 c.c. per kilogram hour) of the 2,520 c.c. injected. Two other animals that received 150 c.c. per kilogram per hour for six hours died on the second day. One of the animals, which three

weeks before had received 125 c.c. per kilogram per hour, put out 92 per cent (138 c.c. per kilogram hour) of the 2,970 c.c. injected, and the other put out 88 per cent (132 c.c. per kilogram hour) of the 2,700 c.c. injected. It would seem that the limit that the rabbit can tolerate is between 125 and 150 c.c. per kilogram per hour. The average output for the three animals was 89 per cent of the amount injected (133.5 c.c. per kilogram hour), or 84 per cent of the body weight of the animal excreted in six hours, a very creditable performance.

In continuing our work with other animals, the dog seemed the one most easily available, and of such size that the total amounts injected would approximate amounts to be used for human injection.

Dog 1, weight 15.5 kg., received 50 c.c. per kilogram per hour for six hours on succeeding days. On the first day it received 4,620 c.c. in six hours and excreted 4,430 c.c. in the same time, an output of 96 per cent (47.6 c.c. per kilogram hour). The following day it received the same amount, but put out only 625 c.c., or 14 per cent (7 c.c. per kilogram hour), in the six-hour period. It gained 4 kg. this day, which it lost in the next three days. Its blood urea was 37 mg. per 100 c.c. before the first injection and 86 mg. before the second injection. The blood urea rose gradually to 233 mg. There were constantly many leucocytes in the urine and casts and erythrocytes after the first day. The phenolsulfonphthalein output fell to 10 per cent on the second day, but rose to 45 per cent the day before death. The animal died the fourth day after the first injection. At necropsy hemorrhages were found in the wall of the stomach and small and large bowel, as well as severe pyelitis with several huge abscesses in the kidneys. The epithelium showed marked vacuolar degeneration, but no necrosis of the epithelium.

Dog 2, weight 8.8 kg., received an intravenous injection of 2,640 c.c. in six hours and excreted 2,370 c.c. (45 c.c. per kilogram hour), or 90 per cent, during the first injection, and 1,875 c.c. (35.5 c.c. per kilogram hour), or 71 per cent, during the second injection the second day following. The animal appeared sick after the second injection and vomited two or three times daily. It died the fourth day after the first injection. Its phenolsulfonphthalein output was 80 per cent before the first injection, 65 per cent after the first, 65 per cent after the second injection, and 59 per cent two days after the second. There were albumin, grade 4, and erythrocytes, grade 4, after the first injection, as well as after the second injection. This animal also showed hemorrhages into the wall of the bowel and wall of the bladder. The kidney showed mild pyelitis, some sclerosis of the glomeruli, and marked changes in the convoluted tubules.

Dog 3, weight 12.6 kg., received two injections of 50 c.c. per kilogram per hour for six hours at an interval of one week. During the first injection it received 3,600 c.c. and excreted 3,000 (41.5 c.c. per kilogram hour), or 83 per cent, and at the second injection it received 3,780 c.c. and excreted 3,040 (40 c.c. per kilogram hour), or 80 per cent.

Dog 4, weight 9 kg., received twenty-eight injections of 50 c.c. per kilogram per hour for six hours. With one exception, when 2,675 c.c. was given, the animal received 2,790 c.c. during each six-hour period. The output varied from

1,830 c.c. during the first injection to 2,365 c.c. during the nineteenth injection, varying in output from 63 to 86 per cent of the amount injected, the average being 80 per cent (40 c.c. per kilogram hour) (Fig. 5). After the twenty-seventh injection the left kidney was removed. Five weeks later the output after injection of 2,790 c.c. was 2,100 c.c. (35.0 c.c. per kilogram per hour), or 75 per cent of the amount injected.

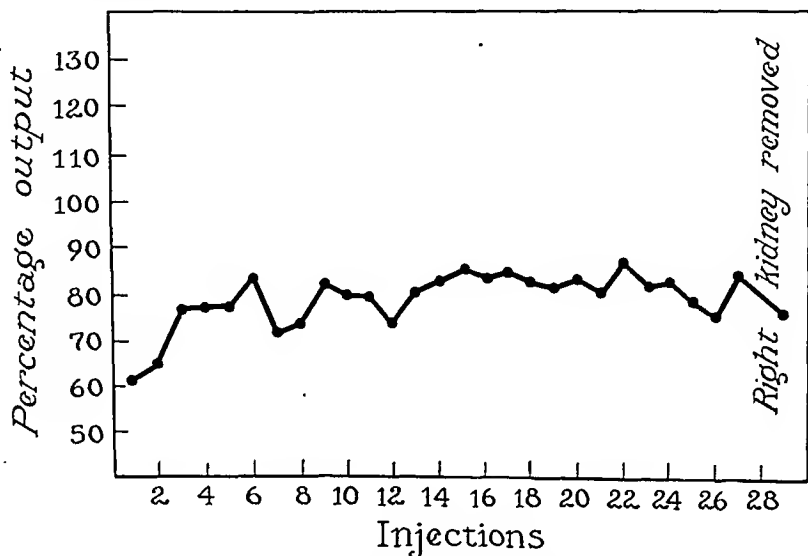


Fig. 5.—Output of urine after intravenous injection of sucrose-Ringer's solution as percentage of the amount injected.

The blood urea was 24 mg. per 100 c.c. before injection; it rose to 59 mg. after the first injection, went down to 33 mg. on the fourth day after the injection, rose again to 60 mg. after the second injection, and went down to 27 mg. on the second day. After this the range was between 18 and 38 mg. per 100 c.c., being 18 mg. and 20 mg. the day of the last two injections.

The phenolsulfonphthalein output was 80 per cent before the first injection; it fell to a trace at the end of twenty-four hours after injection, and was back to 63 per cent after forty-eight hours. It ranged between 58 and 94 per cent, with a reading of 26 per cent after the fifth injection and a trace twenty-four hours after the tenth and twelfth injections (given forty-eight hours after the ninth and eleventh injections).

There was albumin, grade 4, in the urine twenty-four hours after the first injection. The same was true after forty-eight hours. On the third day there was only a trace. Again after the tenth and eleventh injections there was albumin, grade 3, and after the twenty-fourth injection, albumin, grade 2.

Erythrocytes, grade 3, appeared after the first injection, and erythrocytes, grade 1, after the fourth, sixth, and seventh injections. Leucocytes in numbers were present after the first and eleventh injections, and a few after twenty-one injections. Constant catheterization probably accounted for these few cells. Nothing unusual occurred after the twenty-eighth injection, which was given to the dog after removal of the left kidney.

In these dogs as well as in the rabbits, the first injection seems to give rise to the most marked changes in the urinary findings and the phenolsulfonphthalein and urea values. It will be noted that the tenth, twelfth, and twenty-fourth injections were given forty-eight hours after the ninth, eleventh, and twenty-third injections, instead of the usual weekly interval, and this may account for the changes after these injections. On the other hand, it can be said that the twenty-fifth and twenty-seventh injections were given at two-day intervals without any change in the phenolsulfonphthalein output, rise in blood urea, or erythrocytes in the urine.



Fig. 6.—Condition of the kidney of Dog 4 five days after the twenty-seventh injection of sucrose-Ringer's solution ( $\times 125$ )

It is, of course, difficult to say why marked changes are observed after one injection and not after another of the same character. The changes observed were definitely temporary, and even repetition of the same insult produces neither the same acute change nor any permanent damage in the histologic structure or the function of the kidney.

The left kidney of the dog, removed five days after the twenty-seventh injection, showed no changes from the appearance of a normal dog's kidney (Fig. 6). This, together with the fact that the remaining kidney five weeks after left nephrectomy could excrete 75 per cent of the amount injected, shows excellent remaining functional capacity of the animal, whose two kidneys averaged 80 per cent in twenty-seven injections.

#### COMMENT

It would seem that in attempting to find a solution that, when given in large amounts intravenously, produces an equally large diuresis, the first requisite must be safety. Does the solution suggested have this requisite? From the number of injections given, we feel that we can safely say that the epithe-

lium, though markedly changed histologically, seems able to function in the changed condition. There is the second objection that more frequently the most severe reaction comes after the first injection with casts, albumin, and sometimes erythrocytes in the urine. This is, however, only a temporary reaction, and occurs only very occasionally after the later injections. Our object in this experiment is to determine the maximal excreting power of the kidney, so as to remain well within the limit of safety when applying these results to treatment in man.

It seems to us that after the results here obtained, it would be justifiable to use this solution clinically in cases in which there was an emergency in which it was necessary to produce rapid washing out of the urinary passages, as in acute pyelonephritis, to relieve obstruction with or without infection and high blood urea, to prevent ascending infection after operation on the prostate or other lower urinary obstruction, and also to produce good diuresis after a plastic operation on a hydronephrotic kidney.

#### SUMMARY

The rabbit can excrete from 90 to 100 per cent of fluid, representing from 54 to 60 per cent of its body weight, when given in the form of 8 per cent sucrose in half strength Ringer's solution at a rate of 100 c.c. per kilogram per hour for a six-hour period. Such injections may be repeated as often as every other day, with equally effective diuretic effect and no detectable impairment of renal function. A rabbit can excrete 89 per cent (133 c.c. per kilogram hour) of the injected fluid when given at a rate of 150 c.c. per kilogram per hour, a total of 84 per cent of its body weight in six hours, but most of these animals die within forty-eight hours after injection.

The dog can excrete 80 per cent of the fluid given intravenously in the form of 8 per cent sucrose in half strength Ringer's solution when given at a rate of 50 c.c. per kilogram per hour for a six-hour period weekly.

The kidneys in neither animal show any permanent damage after multiple injections, and regain their normal histologic structure in a period of ten days.

The first injection is the one after which the functional capacity of the kidney seems most likely to be lowered, but this lowering is only temporary.

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## VARIATIONS OF SERUM MAGNESIUM IN 52 NORMAL AND 440 PATHOLOGIC PATIENTS\*

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CONSIDERABLE data are being published relative to the variations of serum magnesium in health and disease. Upon examination of this information, it is apparent that too little is known at the present time to report with finality that certain abnormalities seen in man are due to a derangement of magnesium metabolism. A definite tendency toward a hypomagnesemia or hypermagnesemia seems to exist in certain clinical entities, and only after the accumulation of considerable data will it be possible to ascertain whether or not a disturbance in magnesium metabolism may play a role in the etiology of disease. Hence this investigation was undertaken.

### EXPERIMENTAL

Serum magnesium determinations were made on 52 normal and 440 pathologic persons. The normal subjects were medical students and laboratory technicians. The pathologic cases were all hospitalized patients at the time that the blood samples were taken and analyzed. Since ingestion of food has no particular influence on the blood magnesium, no attempt was made to obtain the samples of blood at any particular time in reference to meals. The majority of the determinations were made by the modification of the titan yellow method,<sup>1</sup> yet a few were made by the method described by Briggs.<sup>2</sup>

As can be seen from Table I, the serum magnesium values in the majority of the normal persons (79 per cent) range between 2 and 3 mg. per 100 c.c. The average serum magnesium for the entire group is 2.48 mg. per 100 c.c. This corresponds very closely to the average mean of 2.35 mg. per 100 c.c. of serum, as reported by 17 different investigators (see Table II). In our group only one normal person was found to have a magnesium value slightly below 1.7 mg. We consider, therefore, 1.7 and 3.1 mg. of magnesium per 100 c.c. of serum as the lower and upper limits of normal, respectively.

Nothing unusual is noted in the values obtained from those patients suffering from endocrine disturbances. Our results on diabetic patients support the variability of blood magnesium as already noted by other investigators. In 12.5 per cent of the patients suffering from diabetes mellitus we found a low serum magnesium. This was previously reported by Blumgarten and Rohdenburg.<sup>10</sup> In 15 (26 per cent) of our cases a hypermagnesemia occurred. This confirms the findings of Watchorn and McCance.<sup>5</sup> ✓

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The few patients suffering from toxic thyroid gland and polyglandular disturbances, which we have examined, show that a low blood magnesium may prevail. Blumgarten and Rohdenberg<sup>19</sup> report that 15 of their 45 patients suffering from goiter had a low blood magnesium. Recently, Soffer and co-workers<sup>20</sup> made the interesting observation that the ultrafiltrable magnesium in 31 patients with hyperthyroidism was low (average 64 per cent) when compared to that found in 14 normal persons (average 85.5 per cent).

More definite findings have been obtained in patients suffering from renal and circulatory diseases. The majority of the patients with chronic nephritis, as seen in Table I, reveal an elevated serum magnesium (average 3.56 mg. per 100 c.c.). Most of these patients also had an impairment of kidney function. It is interesting to note that those patients having hypertension associated with definite renal impairment also had an elevated serum magnesium (average 3.90 mg. per 100 c.c.). Hypertensive and arteriosclerotic patients, without apparent renal damage, had a definite though less marked elevation of blood magnesium. This would seem to bear out the inference of Becher and Hamann<sup>21</sup> that the concentration of this ion is increased in hypertension as a result of the associated kidney damage. Numerous reports have appeared in the literature which would indicate that a retention of magnesium is rather common in cases of renal insufficiency.<sup>5, 9, 10, 14, 21-25</sup>

Those patients suffering from vasospastic diseases present a different type of picture. More than 17 per cent of the group have a hypomagnesemia. The group as a whole is somewhat below normal in so far as the mean blood magnesium is concerned (2.12 mg. per 100 c.c. of serum). From these figures it would appear that some relationship may exist between hypomagnesemia and spasticity of the smooth muscles in the blood vessels.

From Table I it may be noted that 23 per cent of the 26 patients suffering from malignant neoplasms have a low serum magnesium. Other investigators<sup>19, 26, 27</sup> have reported similar findings. This ion has on occasion been implicated as one of the etiologic factors in the cause of cancer.<sup>28</sup>

In the patients suffering from hepatic disorders, especially those associated with jaundice, a significant increase in the serum magnesium is noted. Becher and Hamann,<sup>21</sup> Blumgarten and Rohdenburg,<sup>19</sup> and Jung<sup>29</sup> have reported similar findings. In this connection it is interesting to note that Rothman, Meranze, and Meranze<sup>30</sup> found the blood phosphatase to be high in cases of obstructive jaundice. Erdtmann<sup>31</sup> had previously recorded that phosphatase activity is greatly increased by the addition of magnesium.

Far too few cases of infectious diseases have been studied to draw any conclusions from the results. In our patients 3 of the 5 cases of chronic arthritis studied had a low serum magnesium.

The blood of 8 women suffering from toxemia of pregnancy was studied. It seems significant that 3 of these should have a low serum magnesium. One of these patients who had a magnesium concentration below 1 mg. per 100 c.c. of serum also had severe convulsions. Probably the successful use of magnesium sulfate in the treatment of eclampsia<sup>32-35</sup> depends, at least in some cases, upon the elevation of the blood magnesium to the proper physiologic level.

## DETAILED ANALYSIS OF THE SERUM MAGNESIUM

The bold face vertical lines represent the

| CLINICAL DIAGNOSIS  | NO.<br>OF PA-<br>TIENTS | MILLIGRAMS OF MAGNESIUM |                  |                  |                  |                  |                  |                  |                  |                  |
|---|-------------------------|-------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|   |                         | BE-<br>LOW<br>1.0       | 1.0<br>TO<br>1.2 | 1.2<br>TO<br>1.4 | 1.4<br>TO<br>1.6 | 1.6<br>TO<br>1.7 | 1.7<br>TO<br>1.9 | 1.9<br>TO<br>2.1 | 2.1<br>TO<br>2.3 | 2.3<br>TO<br>2.5 |
| Normal individuals  | 52                      | -                       | -                | -                | -                | 1                | 3                | 3                | 8                | 15               |
| 1. Endocrine disorders:   |                         |                         |                  |                  |                  |                  |                  |                  |                  |                  |
| Diabetes mellitus   | 56                      | 2                       | -                | 3                | 1                | 1                | 1                | 2                | 3                | 2                |
| Toxic adenoma of thyroid  | 10                      | 1                       | -                | -                | 1                | 1                | 1                | -                | 1                | 1                |
| Polyglandular disturbances  | 5                       | -                       | -                | -                | 1                | 1                | 1                | -                | -                | -                |
| 2. Circulatory disorders:   |                         |                         |                  |                  |                  |                  |                  |                  |                  |                  |
| Arteriosclerosis  | 15                      | -                       | -                | -                | -                | -                | -                | -                | -                | 1                |
| Hypertension without renal<br>functional impairment   | 65                      | 1                       | -                | -                | -                | 1                | -                | -                | 3                | 5                |
| Hypertension with renal<br>functional impairment  | 23                      | -                       | -                | -                | -                | -                | -                | -                | -                | -                |
| Vasospastic diseases  | 52                      | -                       | -                | 1                | 2                | 6                | 11               | 9                | 10               | 3                |
| 3. Renal disorders:   |                         |                         |                  |                  |                  |                  |                  |                  |                  |                  |
| Chronic nephritis   | 26                      | -                       | -                | -                | -                | 1                | -                | -                | -                | -                |
| Acute nephritis   | 3                       | -                       | -                | -                | -                | -                | 1                | -                | 1                | -                |
| Renal calculi   | 3                       | -                       | -                | -                | -                | -                | -                | -                | -                | -                |
| Urinary infections  | 5                       | -                       | -                | -                | -                | -                | -                | -                | -                | -                |
| Miscellaneous   | 5                       | -                       | -                | -                | -                | 1                | -                | -                | 1                | -                |
| 4. Pulmonary disorders:   |                         |                         |                  |                  |                  |                  |                  |                  |                  |                  |
| Chronic pulmonary infec-<br>tions (including tuber-<br>culosis and pleural infec-<br>tions) | 14                      | 1                       | -                | -                | -                | 1                | 1                | 1                | -                | 1                |
| Pneumonia (lobar)   | 5                       | -                       | -                | -                | -                | -                | -                | 1                | 1                | -                |
| 5. Neoplastic disorders:  |                         |                         |                  |                  |                  |                  |                  |                  |                  |                  |
| Malignant neoplasms   | 26                      | -                       | -                | 3                | 1                | 2                | 2                | 2                | 2                | -                |
| Benign neoplasms  | 5                       | -                       | -                | -                | -                | -                | -                | 1                | 1                | 1                |
| Prostatic hyperplasia   | 15                      | -                       | -                | -                | -                | -                | 2                | 1                | 2                | 2                |
| 6. Hepatic disorders:   |                         |                         |                  |                  |                  |                  |                  |                  |                  |                  |
| Chronic calculus chole-<br>cystitis   | 16                      | 1                       | -                | -                | 2                | 1                | -                | 1                | -                | 2                |
| Chronic noncalculus chole-<br>cystitis  | 13                      | -                       | -                | -                | -                | -                | 2                | 1                | 1                | 1                |
| Acute catarrhal jaundice  | 6                       | -                       | -                | -                | -                | -                | -                | -                | -                | -                |
| Cirrhosis of liver  | 9                       | -                       | -                | 1                | -                | -                | 1                | 2                | -                | 1                |
| 7. Infectious diseases:   |                         |                         |                  |                  |                  |                  |                  |                  |                  |                  |
| Acute rheumatic fever   | 3                       | -                       | -                | -                | -                | -                | -                | -                | -                | -                |
| Chronic arthritis   | 5                       | 1                       | -                | -                | 2                | -                | -                | 1                | -                | 1                |
| Mastoiditis and otitis<br>media   | 3                       | -                       | -                | -                | -                | -                | -                | -                | -                | -                |
| Diseases of the eye   | 4                       | -                       | -                | -                | -                | -                | -                | 1                | -                | -                |
| Superficial abscesses   | 3                       | -                       | 1                | -                | -                | -                | -                | -                | -                | -                |
| 8. Obstetric:   |                         |                         |                  |                  |                  |                  |                  |                  |                  |                  |
| Normal pregnancies  | 4                       | -                       | -                | -                | -                | 1                | -                | 2                | -                | 1                |
| Toxemia of pregnancy  | 8                       | 1                       | 2                | -                | -                | -                | -                | -                | -                | -                |
| Gynecologic diseases  | 7                       | -                       | -                | -                | -                | -                | 2                | 2                | -                | -                |
| 9. Diseases of the central<br>nervous system  | 7                       | -                       | -                | -                | -                | -                | 1                | 1                | -                | 1                |
| 10. Orthopedic surgical prob-<br>lems   | 6                       | 2*                      | -                | -                | -                | 1                | 1                | -                | -                | -                |
| 11. Epilepsy  | 5                       | -                       | 1                | -                | 1                | -                | -                | 1                | 1                | 1                |
| 12. Peptic ulcers   | 8                       | -                       | -                | -                | -                | -                | -                | -                | 1                | 1                |

\*Both were cases of ununited fractures.

†Intra-articular adhesions of right shoulder with beginning ossification of the joint.



I

VALUES IN NORMAL AND PATHOLOGIC PATIENTS

lower and upper limits of the normal range.

| PER 100 C.C. OF SERUM |                  |                  |                  |                  |                  |                  |                  |                  |                  |                  |                  |                  | MEAN |
|-----------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------|
| 2.5<br>TO<br>2.7      | 2.7<br>TO<br>2.9 | 2.9<br>TO<br>3.1 | 3.1<br>TO<br>3.3 | 3.3<br>TO<br>3.5 | 3.5<br>TO<br>3.7 | 3.7<br>TO<br>3.9 | 3.9<br>TO<br>4.1 | 4.1<br>TO<br>4.5 | 4.5<br>TO<br>5.0 | 5.0<br>TO<br>6.0 | 6.0<br>TO<br>7.0 | 7.0<br>TO<br>8.0 |      |
| 13                    | 5                | 3                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.48 |
| 5                     | 12               | 9                | 8                | 3                | 3                | 1                | -                | -                | -                | -                | -                | -                | 2.69 |
| 2                     | 1                | 1                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.51 |
| 1                     | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.07 |
| 3                     | 6                | 2                | 2                | -                | -                | -                | -                | 1                | -                | -                | -                | -                | 3.05 |
| 8                     | 15               | 16               | 9                | 1                | 2                | 2                | 1                | 1                | -                | -                | -                | -                | 2.88 |
| 1                     | 2                | 5                | 1                | 1                | -                | 1                | 5                | 3                | 1                | 2                | -                | 1                | 3.90 |
| 3                     | 4                | 2                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.12 |
| 4                     | 1                | 3                | 3                | -                | 2                | 6                | 2                | 2                | 1                | 1                | -                | -                | 3.56 |
| 1                     | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.31 |
| -                     | 1                | 1                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 3.00 |
| -                     | 2                | 2                | -                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | 2.97 |
| 1                     | 1                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.50 |
| 1                     | 2                | 1                | 1                | 1                | 2                | 1                | -                | -                | -                | -                | -                | -                | 2.66 |
| 2                     | -                | -                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.49 |
| 1                     | 4                | 2                | 5                | 1                | -                | 1                | -                | -                | -                | -                | -                | -                | 2.10 |
| -                     | 1                | -                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.51 |
| 2                     | 1                | 3                | -                | 1                | 1                | -                | -                | -                | -                | -                | -                | -                | 2.61 |
| -                     | 2                | 1                | -                | 2                | 1                | 1                | -                | 2                | -                | -                | -                | -                | 2.73 |
| -                     | 2                | 3                | 2                | -                | -                | -                | 1                | -                | -                | -                | -                | -                | 2.73 |
| -                     | -                | 2                | -                | 1                | 1                | 1                | -                | -                | 1                | -                | -                | -                | 3.56 |
| -                     | -                | 1                | 1                | 1                | 1                | -                | -                | -                | -                | -                | -                | -                | 2.48 |
| -                     | 1                | -                | -                | 1                | 1                | -                | -                | -                | -                | -                | -                | -                | 3.33 |
| -                     | 1                | 1                | -                | -                | 1                | -                | -                | -                | -                | -                | -                | -                | 1.71 |
| -                     | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 3.16 |
| 2                     | -                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.52 |
| -                     | 1                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.31 |
| -                     | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.28 |
| -                     | 1                | 1                | 2                | -                | -                | 1                | -                | -                | -                | -                | -                | -                | 2.38 |
| -                     | 1                | 1                | -                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | 2.45 |
| -                     | 2                | 1                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 2.68 |
| -                     | -                | -                | -                | 1                | -                | -                | -                | 1†               | -                | -                | -                | -                | 2.17 |
| -                     | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | -                | 1.85 |
| 2                     | 1                | 1                | -                | -                | 1                | 1                | -                | -                | -                | -                | -                | -                | 2.80 |

We studied 3 cases with orthopedic surgical conditions. In 2 of these a marked hypomagnesemia and in one a marked hypermagnesemia occurred. The 2 patients with hypomagnesemia were cases of ununited fractures of the patella and tibia, and their serum magnesium values were 0.96 and 0.80 mg. per 100 c.c. of serum, respectively. Further study revealed that the latter patient required an amputation of the limb one year after the above blood study was made. The patient with a hypermagnesemia of 4.4 mg. per 100 c.c. of serum suffered from intra-articular adhesions of the right shoulder with beginning ossification of the joint.

TABLE II  
CONCENTRATION OF SERUM MAGNESIUM IN NORMAL PERSONS

| AUTHORS                                | PRECIPITATING AGENT | METHOD       | RANGE OF VALUE | MEAN |
|--|---------------------|--------------|----------------|------|
| Briggs <sup>2</sup>                    | Phosphate           | Colorimetric | 2.23-2.50      |      |
| Kramer and Tisdall <sup>3</sup>        | Phosphate           | Colorimetric | 1.80-2.30      | 2.10 |
| Bogert and Plass <sup>4</sup>          | Phosphate           | Colorimetric | 1.90-2.70      | 2.30 |
| Wathehorn and McCance <sup>5</sup>     | Phosphate           | Colorimetric | 2.30-2.66      | 2.48 |
| Becher <sup>6</sup>                    | Phosphate           | Colorimetric | 1.80-2.30      |      |
| Wacker and Fahrig <sup>7</sup>         | Phosphate           | Colorimetric | 2.00-2.97      | 2.40 |
| Copes <sup>8</sup>                     | Phosphate           | Colorimetric | 1.82-2.63      | 2.06 |
| Walker and Walker <sup>9</sup>         | Phosphate           | Colorimetric | 1.60-3.00      | 2.20 |
| Brookfield <sup>10</sup>               | Phosphate           | Colorimetric | 1.89-2.19      | 2.04 |
| Bomskov <sup>11</sup>                  | Hydroxyquinoline    | Bromination  | 1.70-2.60      |      |
| Greenberg and co-workers <sup>12</sup> | Hydroxyquinoline    | Bromination  | 2.00-3.66      | 2.74 |
| Velluz and Velluz <sup>13</sup>        | Hydroxyquinoline    | Bromination  | 1.60-2.40      | 2.00 |
| Raices <sup>14</sup>                   | Hydroxyquinoline    | Bromination  | 1.69-3.00      | 2.44 |
| Hoffman <sup>15</sup>                  | Hydroxyquinoline    | Colorimetric | 1.90-2.50      | 2.18 |
| Hirschfelder and Haury <sup>16</sup>   | Titan yellow        | Colorimetric | 1.80-2.40      | 2.11 |
| Haury <sup>17</sup>                    | Titan yellow        | Colorimetric | 1.70-3.10      | 2.33 |
| Wolfis <sup>18</sup>                   | Titan yellow        | Colorimetric | 2.90-4.00      | 3.61 |

\*For whole blood.

The 3 cases suggest to us the possibility that a deficiency of magnesium may be partly responsible for the delayed formation of callus. Conversely, a hypermagnesemia may play some etiologic role in the production of intra-articular adhesion and ossification.

In epilepsy a deficiency of magnesium was found in 2 of the 5 patients examined. This is in close agreement with the findings of Denis and Talbot,<sup>26</sup> Blumgarten and Rohdenburg,<sup>19</sup> Wolf,<sup>27</sup> and Hirschfelder and Haury.<sup>38</sup> This is not surprising since the establishment of the close relationship between magnesium deficiency and convulsions.<sup>39-43</sup> However, Greenberg and Aird<sup>44</sup> found the blood serum magnesium essentially normal in 22 patients with essential epilepsy.

#### SUMMARY

The results of serum magnesium determinations made in our laboratory on 52 normal and 440 pathologic patients are reported and the results are briefly discussed.

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## THE ABSORPTION OF STILBESTROL AND THEELIN FROM CYSTS OF SESAME AND PEANUT OILS\*

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A LARGE number of animals treated in various ways with stilbestrol were autopsied during the last two years. Cysts were always found in the rats given oil by intramuscular injections and usually found after subcutaneous injections. The estrogenic content of the oil from many of these cysts was studied.

### METHODS

The procedure followed was an outgrowth from experiments designed to test the minimal dose of stilbestrol. Failure to obtain consistent results with stilbestrol in oil led to a more careful study of the absorption of the oil. Data were taken from a total of 260 albino rats. Forty female rats used for minimal dose testing formed the beginning of these experiments. This group was given 0.3 c.c. of sesame oil in three doses of 0.1 c.c. each, 20 being injected intramuscularly and 20 in the subcutaneous tissue of the flank. A second group of 30 castrated mature female rats were injected subcutaneously in the back and 90 male rats intramuscularly in the hind legs with 1.0 mg. of stilbestrol in 1.0 c.c. of sesame oil. For the purpose of comparing theelin with stilbestrol, an additional 60 female rats were injected intramuscularly with 1.0 c.c. of either peanut oil or sesame oil containing 1.0 mg. of stilbestrol or theelin, and estrous cycles were followed for a period of thirty days (Fig. 4). Forty males injected intramuscularly with theelin were also added for cyst studies (Fig. 3). The needle was inserted to a depth of about 2 cm., and without further manipulation the 1.0 c.c. of the oil was deposited in one globule. The skin was pinched to

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prevent leakage of the oil. Some of these rats were autopsied each day for a period of forty days, and thereafter at various times up to a period of nine months after the injections were made. The oil from the cysts of each leg was collected separately, shaken in 5.0 c.c. of a salt solution (0.9 per cent), and the resulting oil emulsion given intraperitoneally to a castrated female rat. The estrous cycles were taken twice daily by the pipette method<sup>1</sup> as long as the rats remained in estrus.

### RESULTS

The 40 rats that were given doses of 0.1 c.c. of sesame oil all showed multiple cyst formation in the muscles and the subcutaneous tissue of the flank. These cysts were usually small, 1 to 3 mm. in diameter, and full of clear oil.



Fig. 1.—A castrated female rat used for minimal dose testing of stilbestrol and given several subcutaneous injections of sesame oil in amounts of 0.1 c.c. each. The cysts of oil are typical of rats injected with sesame oil.

The cyst wall was firm and could easily be dissected free (Figs. 1 and 2). The quantity of stilbestrol injected was so small that tests for its estrogenic content from the encysted oil were not made. Only occasionally were large cysts present in these rats. Likewise the 30 rats injected with 1.0 c.c. of oil subcutaneously in the back usually showed similar cysts, but these were less abundant, indicating that only part of the oil as it gravitated downward from the back had remained in one area long enough to become encysted. At autopsy the 90 male rats which were injected intramuscularly with 1.0 c.c. of sesame oil showed larger cysts than the previously mentioned groups, and from these sufficient oil for testing could be obtained. The amount of oil collected from the cysts in the leg varied from 0.2 to 1.0 c.c. and diminished somewhat as postinjection time increased (Fig. 3). The cyst wall was found as early as the third day after the injections were made, and after five or six days it was always present. Up to at least a month after the cysts were formed, the oil was not always clear,

being frequently creamy to cheesy in consistency, and white in color. Although observations on cysts of longer duration are less in number, they indicate that the oil may be entirely clear, or clear with white, cheesy masses floating in it for at least nine months after treatment.

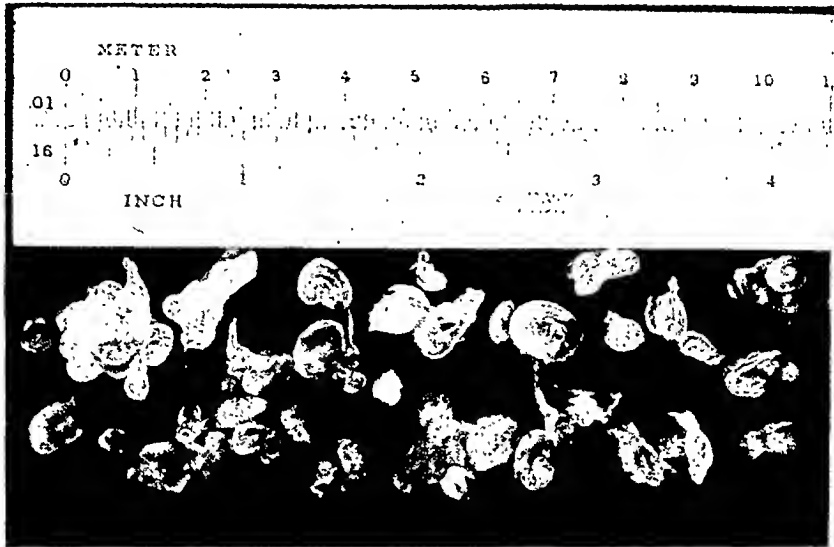


Fig. 2.—Cysts containing sesame oil dissected after being in the subcutaneous tissue and muscles of rats for varying periods up to nine months.

Fig. 3 depicts the activity of the stilbestrol remaining in the cystic oil after one to thirty-six days in the muscles. This curve, as first constructed from 60 tests, was very irregular. Since this might be expected from scattered data, an additional 30 tests were made at the irregular points. Therefore, it is fairly regular and shows that the percentage of rats giving positive vaginal smears diminish from 82 per cent on the first to third day to zero percentage at twenty-three days. One animal did not react when given 0.4 c.e. of cyst oil on the third day. This is difficult to evaluate, but since the slope of the curve is downward at four to six days and continues downward thereafter, these negative cases are not incidental to the technique of testing but rather indicate that stilbestrol was not present in sufficient amounts to give a positive vaginal reaction. Only one positive test was obtained after the twenty-first day, and this was from oil after twenty-nine days in the muscle (Fig. 3).

In a similar manner, 40 males were injected with peanut oil containing theelin to determine the theelin remaining in the cysts. Peanut oil, like sesame oil, became readily encysted. Theelin disappeared from these oil cysts sooner than the stilbestrol, the curve dropping sharply from the second to the eighth day. After the eleventh day further positive reactions were not found (Fig. 3). The difference between the amounts of stilbestrol and theelin remaining in the cystic oil may be explained by solubility. The theelin being more soluble in water was more readily removed by the tissue fluids surrounding the cysts, whereas the stilbestrol, although very soluble in oil, was insoluble in water.

The duration of estrus after a single injection of 1.0 c.c. of peanut oil containing 1.0 mg. of stilbestrol or theelin was determined in adult female rats. Fig. 4 shows the percentage of rats in estrus plotted against time in days. It is clearly seen that the curve for the stilbestrol-treated rats dropped slowly from the eighth to the twenty-first day, whereas the comparable curve for theelin absorption was considerably below this, having started downward

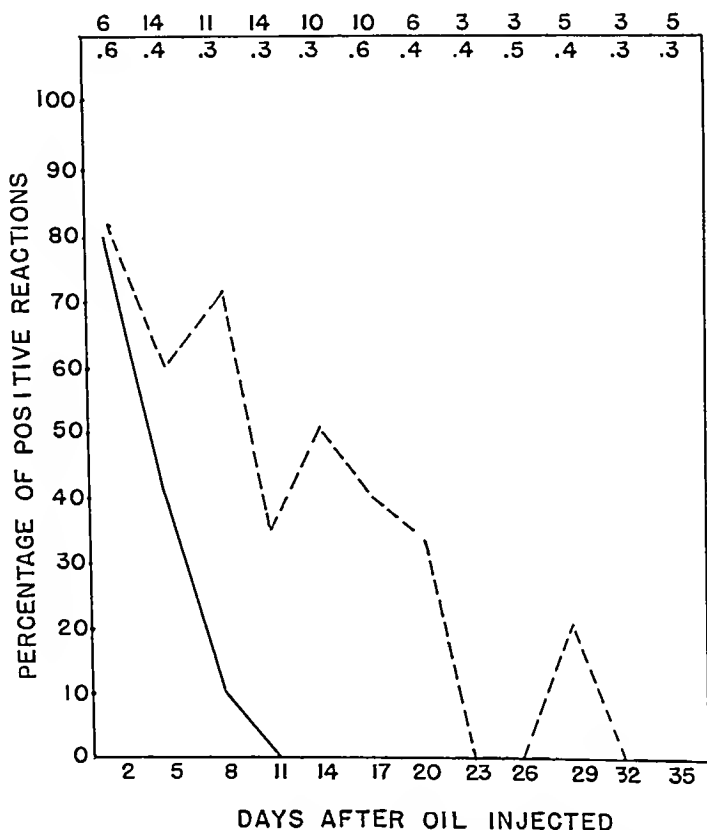


Fig. 3.—The per cent of castrated rats showing estrus after a single injection of oil from cysts containing stilbestrol (upper curve, 90 rats) and theelin (lower curve, 40 rats). Abscissa (lower), time in days that the oil was in the muscles of the donor rats. Abscissa (upper, below line), mean amounts of oil in cubic centimeters recovered each three-day period from the original 1.0 c.c. of sesame oil injected in the muscles of the donor rats. These figures also represent the amount of oil reinjected into the 90 test rats forming the upper curve. Abscissa (upper, above line), the 90 rats used for the stilbestrol curve subdivided to show the number of tests made at three-day intervals. The 90 male rats received 1 c.c. of sesame oil containing 1.0 mg. of stilbestrol. The 40 males received 1 c.c. of peanut oil containing 1.0 mg. of theelin. Both male groups were treated once only; the 1 c.c. of oil being injected in one globule in the muscles.

sharply on the fifth day and reached zero by the fourteenth day, thus indicating the more rapid absorption of theelin. These curves also conform in time, and to a large extent attest the validity of the estrous curves obtained with the oil from the cysts (compare Fig. 3 with Fig. 4). For the purpose of comparison the absorption of stilbestrol from both peanut oil and sesame oil is shown in Fig. 4. The two curves are identical until the ninth day and are only slightly separated thereafter, as indicated by the dotted line in Fig. 4.

## DISCUSSION

The multiple cysts found in the subcutaneous tissue and muscles of rats given minimal doses of stilbestrol in a total of only 0.3 c.c. of sesame oil (Fig. 1) show that oil itself is poorly absorbed and leads to lack of confidence that stilbestrol could be uniformly absorbed through the fairly thick walls of the cysts. These oil pockets were found to be encysted by the fifth day, and the cyst wall appeared to become thicker and more durable for some time thereafter. Further evidence that sesame or peanut oil is an inadequate vehicle for stilbestrol was also obtained with larger doses of stilbestrol because they show that in some cases

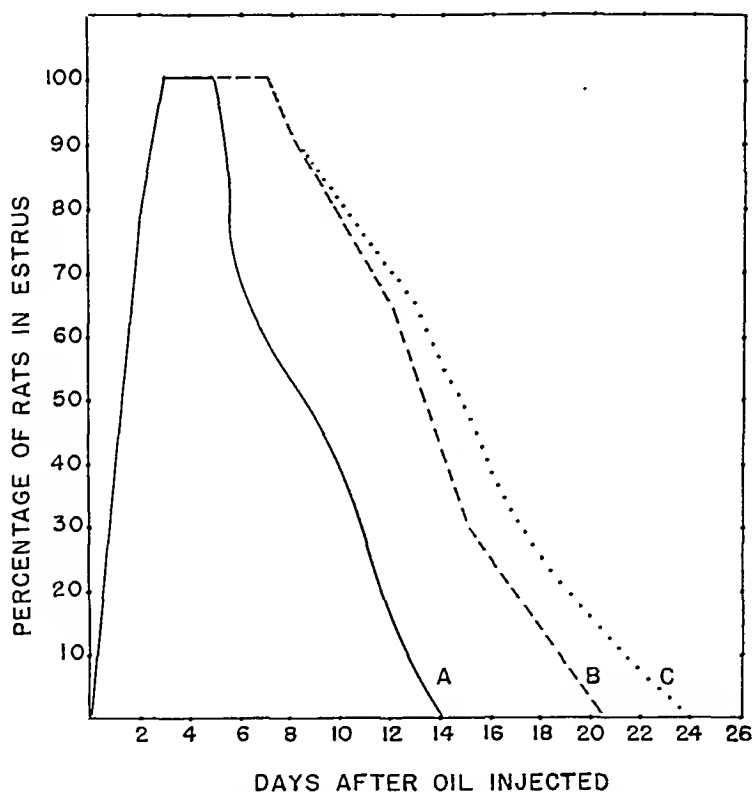


Fig. 4.—Lower curve, A, the per cent of rats in estrus each day after 1.0 mg. of theelin in peanut oil was given intramuscularly. Upper curve, B, after 1.0 mg. of stilbestrol in 1.0 c.c. of peanut oil was injected intramuscularly. Dotted line, C, same in sesame oil. Twenty castrated rats were used in each of the three groups.

all the stilbestrol (1.0 mg.) may be absorbed in three days, while in other rats given identical treatment, the stilbestrol was still present after being in the muscles for twenty days (Fig. 3). Such a range of time for complete absorption probably would be less for smaller doses; but the uncertainty as to when all the stilbestrol would be absorbed makes minimal dose testing with oil solutions confusing.

After more than 150 tests were carefully studied, hope of determining the minimal dose of crystalline stilbestrol in oil was abandoned. Much confusion still exists in the literature concerning oil and aqueous solutions of estrogens. Korenchevsky, Dennison, and Schalit<sup>2</sup> found that oil remained several days after



intramuscular injections and discussed the uncertainty of oil absorption. Even more impressing data were obtained by Deanesly and Parkes,<sup>3</sup> who found estrin in castor oil after fifteen days in the subcutaneous tissues of mice, and suggested that oil is a highly unsuitable medium for quantitative assay. On the other hand, Bülbring and Burn,<sup>4</sup> using the weight of the uterus as an index of estrone activity, felt that their error in testing was small. The sensitivity of the uterus to small amounts of estrone in the blood<sup>5,6</sup> and the duration of the uterine growth test, which is usually beyond the time required for vaginal smear readings, give evidence that this test may be quite reliable for oil solutions. Also, estrogens in oil solutions deteriorate less readily than in aqueous solutions.<sup>7</sup> These observations extended by these data led to the conclusion that oil was a vehicle from which absorption not only was slow and variable but also had the disadvantage of almost always becoming encapsulated and remaining in a cystic state for many months (up to nine months, Fig. 2; for as long as fifteen months not illustrated).

Oils differ in their ability to form cysts. Castor oil produced more cysts than sesame oil, and the latter produced more than olive oil.<sup>3</sup> The present experiments indicate a similarity between sesame oil and peanut oil, judging by their ability to form cysts and by the duration of estrus induced by the stilbestrol remaining in these oil cysts (Fig. 4). Yet the wall of the sesame oil cysts appeared thicker than the wall of the cysts usually obtained with peanut oil.

#### SUMMARY

The formation of cysts following injections of sesame oil and peanut oil was observed in all rats (210) injected intramuscularly and was usually present in rats (50) injected subcutaneously. Oil obtained from the cysts was tested on castrated rats for stilbestrol and theelin. The curves for these tests showed that in some cases all the estrogenic activity was gone by the third day, but in a few rats some stilbestrol remained in the cysts even after twenty days. This is in marked contrast to theelin, in which case the time of retention was reduced to approximately nine days. These cysts, containing oil, remained in the tissues for many months.

Castrated rats were given 1.0 mg. of stilbestrol or theelin in 1.0 c.c. of peanut oil, and the estrous cycles were taken daily. These also confirmed the slow absorption of stilbestrol compared with theelin after a single oil injection.

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# HISTAMINASE

## FURTHER LABORATORY STUDIES

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**H**ISTAMINASE is the term applied to the histamine-inactivating property of aqueous extracts of certain animal body tissues. The properties and actions of histaminase closely resemble those of the enzymes, thus tentatively placing this "substance" in the category of enzymes or ferments. The property was first described by Eustis,<sup>1</sup> who reported inactivation of histamine with the pulp from the liver of a turkey buzzard. Most of the original research upon the characteristics of histaminase is described in the excellent papers of Best,<sup>2</sup> Best and McHenry,<sup>3</sup> and McHenry and Gavin.<sup>4-7</sup> Their exhaustive research has been the basic and guiding principle in this work and in previous work I have done. In the previous publication<sup>8</sup> there was described the method of preparation, crude standardization, titration, and some clinical application of histaminase.

### METHOD OF PREPARATION AND TITRATION

As previously described,<sup>8</sup> hog kidney, after mincing, was defatted with acetone, dried with ether, and finely ground. The powder was extracted with phosphate buffer at pH 7.2 under toluene overnight in the incubator at 37° C. All the titrations and tests were performed with my method on my own left forearm, as previously described (except the experiments with trypsin). However, by further refinement, it was possible to arrive at an almost exact determination of the potency. The method follows: The sensitivity of the skin to histamine was first determined. One-tenth cubic centimeter of increasing dilutions of histamine phosphate was injected intracutaneously, and the size of the resulting wheals was measured and recorded.

| CONCENTRATION | WHEELS IN MM. |
|---------------|---------------|
| 1:10,000      | 18            |
| 1:100,000     | 17            |
| 1:1,000,000   | 15            |
| 1:10,000,000  | 12            |
| 1:100,000,000 | 9             |

Hence one could consistently determine one part in five million, or 0.05 gamma of histamine phosphate injected. Thus the skin is less sensitive than the hen's ileum,<sup>9</sup> which can detect 0.01 gamma per cubic centimeter. In actual titration small Florence flasks were used. Into each flask measured amounts of histaminase solution in decreasing quantities were placed, e.g., 1 c.c. solution, 0.5 c.c. solution, 0.25 c.c. solution, etc. To each flask was then added 0.25 mg., or 0.25 c.c., of 1:1,000 histamine phosphate. The volume in each flask was made

up to 10 c.c. with phosphate buffer at pH 7.2. Since all the histamine was taken from one ampoule, only one intracutaneous test (from the mixture in any one flask) was necessary to determine the activity of the histamine. One-tenth cubic centimeter of the mixture from any one flask was injected intracutaneously, and the size of the wheal was measured and recorded. The concentration of the histamine was 1:40,000, and the size of the wheal was usually from 16 to 18 mm. The flasks were then incubated at 37° C. under toluene for twenty-four hours. Starting with the mixture containing the largest amount of histaminase solution, 0.1 c.c. was injected intracutaneously from each flask until one of the sites produced a wheal of over 10 mm. The potency was fixed at the point between the injection which failed to produce a wheal and the one which just produced a wheal. By definition, a unit of histaminase is the quantity of enzyme that inactivates 1 mg. of histamine during twenty-four hours' incubation at 37° C. in a phosphate buffer solution of pH 7. Hence the expression of unitage may vary, depending on whether histamine base, histamine hydrochloride, or histamine phosphate is used as a standard. One milligram of histamine base actually contains 1 mg. of histamine, whereas 1 mg. of histamine hydrochloride involves 0.6 mg. of histamine and 1 mg. of histamine phosphate produces only 0.3 mg. of histamine. Therefore, the actual histamine ratio, according to molecular weights, is 3:2:1, respectively. In this work, since histamine phosphate was used, it is necessary to divide the units expressed by 2 in order to obtain unitage against histamine hydrochloride and by 3 in order to obtain unitage as represented by histamine base. In the future it might be expedient to express all units in relation to histamine base.

According to McHenry and Gavin,<sup>4</sup> extraction is best done by extracting one part of kidney powder with 18 parts of phosphate buffer. The solution is filtered and the powder is again extracted with a similar amount of phosphate buffer. In their procedure the filtrates were combined. This procedure was followed, but each of the filtrates was tested separately. The results obtained are indicated in Table I.

TABLE I  
(Nitrogen is expressed as milligrams per cubic centimeter)

| EXTRACTION | COLOR        | POTENCY                            | PROTEIN NITROGEN | N.P.N. | TOTAL N |
|------------|--------------|------------------------------------|------------------|--------|---------|
| First      | Amber        | Over 10 units per cubic centimeter | 0.90             | 0.29   | 1.19    |
| Second     | Light yellow | None                               | 0.24             | 0.86   | 1.10    |

The following conclusions were drawn:

1. The potency is in a general way directly proportional to the depth of color of the original solution.
2. The second extraction had very little or no potency. It was, therefore, discarded as a step in the procedure.
3. The potency is related to the protein nitrogen fraction. The original extraction was finally modified so that each gram of powder was extracted with only 10 c.c. of phosphate buffer. By this method original solutions (filtrates), having from 5 to 75 units per cubic centimeter (average 12 units per cubic centimeter), were obtained.

## PROPERTIES OF ORIGINAL SOLUTION (FIRST EXTRACTION)

I. The original solution can be clarified and sterilized by Seitz filtration without immediate loss in potency. It is clear, light to dark amber in color, has a strong odor of fresh animal tissue infusion, and is fairly stable at icebox temperature for about one to two months. However, solutions are rather unstable even at these temperatures, developing turbidities, flocculations, and precipitates, and generally losing a maximum of 50 per cent of their potency in from two to three months. One solution kept in the icebox for sixteen months developed a turbidity but maintained about 90 per cent of its original potency. Nevertheless, the solutions are sufficiently stable when fresh (about one month) for various in vitro experiments and for clinical use by intramuscular injection.

II. The solution is rather irritating and produces a distinct smart and pain on intramuscular injection, lasting ten minutes. An expedient maximum dose of 10 c.c. can be injected intramuscularly (average 120 units). Occasionally, one encounters the phenomenon described by Greenbaum<sup>10</sup> of an intense local reaction coming on in a few hours, but this is no contraindication to continued treatment. In one case a severe asthmatic attack was induced in ten minutes, but further injections failed to produce attacks in the same case.

III. The solution can be concentrated to a dry tan powder, without loss of potency, by fanning in a cellophane bag. This dried powder is more permanently stable than the solution and is readily soluble in water. It is quite possible to prepare and store this powder aseptically in ampoules by means of the Adtecac,<sup>11</sup> Lyophile,<sup>12</sup> Cryochem<sup>13</sup> process, or by some similar vacuum-refrigeration-concentration process. Due to lack of suitable equipment these latter procedures were not carried out.

IV. The effects of heat, hydrogen-ion concentration, and tryptic digestion confirm the report of McHenry and Gavin.<sup>4</sup> If the histamine-histaminase mixture was adjusted to a pH of 5.0 or less previous to incubation, the histaminase was irreversibly inactivated. In the tryptic digestion experiments the trypsin used had an index or activity of 3.3 by Gross' method.<sup>14</sup> A little of this trypsin was added to a solution of histaminase having a potency of 2 units per cubic centimeter and stored at icebox temperatures. An untreated control histaminase solution was similarly stored. After a few days the trypsin-treated histaminase and the control were incubated with histamine, and the titration was carried out on the virgin guinea pig uterine strips in the Dale bath. The usual skin titration was not carried out because injected trypsin has properties similar to those of snake venom.<sup>15</sup> The following results were found:

1. Trypsin solution itself did not stimulate the guinea pig uterus.
2. Trypsin-treated histaminase did not stimulate the uterine strip.
3. The control mixture (after incubation) did not cause contraction.
4. The histamine trypsin-treated histaminase mixture (after incubation) caused a marked contraction of the uterine strip.

This showed that the histamine in result 4 was not destroyed and that the trypsin had inactivated the histaminase even at icebox temperatures. Any temperature of over 60° C. inactivated histaminase irreversibly.

V. Attempts to purify the solution by ultrafiltration were not successful. A potent amber histaminase solution, containing 7 units per cubic centimeter, was ultrafiltered with nitrogen pressure through two layers of No. 600 cellophane. While the pH of the solution was 7.2, the clear Nile green ultrafiltrate had a pH over 8.0.

The green ultrafiltrate did not possess any potency and was protein free. The material left on the cellophane was dark brown in color, did not redissolve easily, contained a lot of protein, and had a potency of only 3.5 units per cubic centimeter. Dialysis experiments showed failure of the active material to pass through the cellophane bag into the external buffer.

VI. Attempts to purify the enzyme by ammonium sulfate precipitation, followed by ice acetone extraction, in most instances produced a total loss of potency.

VII. The concentration of histaminase in beef and lamb kidneys is much less than in hog kidneys. Repeated attempts showed a maximum value of 1 unit per 20 c.c. in beef kidney solution, and 1 unit per 3 c.c. in lamb kidney solution. Both of these solutions were light yellow in color.

VIII. In evaluating the potency of hog kidney flesh autolysates (without defatting), freshly ground hog kidneys were allowed to autolyze under toluene in the incubator at 37° C. for from seven to fourteen days. It was found that the histaminase potency was always less than that obtained from the kidney powder. For example, one batch of kidney flesh produced solutions having a potency of 2 units per cubic centimeter. The solution obtained from powder from the same batch had a potency of from 5 to 25 units per cubic centimeter.

IX. As a demonstration of the enzymatic property of histaminase, a solution having a potency of about 1 unit per cubic centimeter was incubated with 1 mg. of histamine phosphate for twenty-four hours and showed the usual slight cloudiness. Skin test showed that histamine had been destroyed. The mixture was clarified by filtration, and another 1 mg. of histamine phosphate was added. After twenty-four hours' incubation, the histamine was found to be inactivated. A further milligram of histamine was added, and in twenty-four hours it was found to be destroyed. This type of reaction is rather characteristic of enzymes, as contrasted with purely chemical reactions.<sup>16</sup>

X. The effects of histaminase and histamine on the human skin and organism, as revealed in this study, may be of some interest. Eight batches of hog kidney powder and one batch each of lamb and beef kidney powder were prepared. Each batch usually contained about 200 Gm. of dried powder. From each batch about five lots of solution were prepared. This made a minimum of 50 lots of solution in a period of eight months. Each lot had an average number of 10 intracutaneous tests done. Hence, over 500 tests with histamine, histaminase, and mixtures of these were done on my left forearm. At no time was there any indication of any local refractoriness to histamine. Nor was there any evidence of increased sensitivity. The same site could be reinjected at any time from fifteen minutes to months after the first injections and still respond with a marked wheal. For example, a fresh site was injected with 0.1 c.c. of 1:10,000 histamine and a wheal of 18 mm. was developed. In about thirty minutes, when the flare was subsiding and the edges of the wheal were

less pronounced, 0.1 c.c. of 1:1,000 histamine phosphate was injected into the same puncture. In a few minutes a larger wheal with pseudopods developed. During the injections for over eight months it was natural that a large amount of histaminase would be injected and absorbed. This had no effect whatsoever on the subsequent histamine skin reactions. Two further experiments were performed:

(1) A potent histaminase solution containing 5 units per cubic centimeter, was injected intracutaneously in three sites, 0.3 c.c. being used at each site. Tests with 1:10,000 histamine phosphate in fifteen minutes, four hours, and twenty-four hours (one site at a time) produced prompt and typical histamine wheals.

(2) A highly potent histaminase solution, containing 15 units per cubic centimeter, was injected in two sites, 1 c.c. being injected intracutaneously at each site. Eighteen hours later one site was tested with 1:40,000 histamine phosphate and produced a wheal of 18 mm.; the other site was tested with 1:1,000 histamine phosphate and produced a wheal of 25 mm.

On several occasions after extensive skin testing, there was some lassitude, back pain, and axillary tenderness. Part of this may have been due to the protein injected. On two occasions there was a neuritis involving the left shoulder and arm. The first was relieved by cessations of the injections and short wave diathermy. The second neuritis responded very promptly to  $\frac{3}{4}$  grain of oral propadrine. Despite the numerous hog protein injections, no sensitivity to pork developed.

#### DISCUSSION

The field of enzyme chemistry has made extraordinary advances in the past ten years. Organic or "living" enzymes are extremely labile substances sensitive to heat, cold, temperature gradients, hydrogen-ion concentration, electrolytic and colloidal concentrations, and many other physical and chemical factors. In order to secure sufficient final yields of high potency and purity, one must begin with hundreds of grams of original material.<sup>17</sup> Enzyme chemists believe that they have recently isolated some of the enzymes in pure crystalline form after many years of intensive research.<sup>17</sup> The most refined present preparations of histaminase are as yet far from the pure state attained for these other enzymes. The histaminase solutions used for injections are conglomerations of numerous aqueous extractives of hog kidney and intestinal mucosa. Separation of proteins in these solutions is quite incomplete, and various enzymes, hormones, salts, and inorganic materials in the mixture have not yet been completely investigated. Therefore, though the mixture inactivates histamine, we do not know as yet the reason for its reported beneficial effects.<sup>18</sup> It must be realized that when the solution is given by injection, a large amount of nonspecific protein is given. In 10 c.c. there are about 9 mg. of protein nitrogen. Its beneficial effects by mouth (considering only the histaminase) are inexplicable in view of the *in vitro* experiments with tryptic digestion and the effect of pH below 5.0.

The enzymatic activity of solutions of histaminase seems definitely linked to the protein. Any attempt to remove all the protein results in inactivation

of the enzyme. This is further evidence that we are dealing with an enzyme in accordance with the present-day concept that organic enzymes are proteins or are closely allied to proteins.<sup>16, 17</sup>

Although different persons have varying sensitivities to intrautaneous histamine, most of them have approximately the same sensitivity as described in this study. Hence, a method of titration of histaminase potency in human beings, using suitably titrated and standardized reeompensed donors, is offered for clinical trial. The method is apparently sufficiently sensitive to meet all but the most exacting requirements. By using histamine from the same sterile lot in all titrations, the number of control injections can be reduced.

It was found that even though a batch of kidney powder was thoroughly mixed, different lots of solution from the batch extracted under exactly the same conditions would have different potencies. Therefore, it is inadvisable to test only one lot at a time in evaluating a batch of powder. Each lot of solution must be titrated separately.

The kidney histaminase variation in members of a species, determined by McHenry and Gavin,<sup>6</sup> is compared with my findings in Table II.

TABLE II

(Figures represent milligrams of histamine base inactivated by 1 Gm. of kidney flesh)

| ANIMAL | MC HENRY AND GAVIN | THIS WORK    |
|--------|--------------------|--------------|
| Sheep  | 3.2                | 0.3 (lamb)   |
| Ox     | 1.6                | 0.16 (steer) |
| Hog    | 3.6                | 10.0 average |

It should be noted that only a small number of batches (one each) of lamb and beef kidney flesh and powder was prepared. Therefore, figures for lamb and steer may not be fully representative.

It has been found<sup>4</sup> that there is a latent period *in vitro* before any inactivation of histamine occurs. This enzymatic property of latency is further demonstrated in the experiments in which skin sites heavily inoculated with potent histaminase solution failed to prevent the whealing reaction of histamine. This lack of "protection" may have some clinical significance.

A word of caution concerning the incubation during titration is in order. During the course of the investigation a peculiar property of histamine was observed. If 1 mg. of histamine phosphate (1 c.c. of 1:1,000) was added to 9 c.c. of phosphate buffer at pH 7.2 and tested intrautaneously, a wheal of 18 to 20 mm. was obtained. If the flask was then incubated at 37° C. for twenty-four hours *without* added toluene, the fluid would become somewhat cloudy. A skin test now would frequently show no histamine effect (lack of whealing), and the guinea pig uterus in the Dale bath would not respond. However, if a thin layer of toluene was added to the histamine in phosphate buffer previous to incubation, the solution would preserve its clarity and also its whealing effect indefinitely. The culture from the cloudy solution showed a gram-positive bacillus. Undoubtedly, the described destructive effect on histamine was due to the action of these air bacilli upon it.

Another paper will deal with the clinical results attained by the injection of potent histaminase solutions.<sup>19</sup>

## SUMMARY

Further laboratory studies in the preparation, titration, properties, and purification of the enzyme-system histaminase are reported. A more accurate method of titration on human skin is described.

The physical properties of phosphate buffer solutions of histaminase and the effects of heat, pH, and trypsin are outlined. Several methods of purification and concentration are evaluated.

Further evidence that histaminase is an enzyme is adduced.

Some of the local and general effects of histamine and histaminase used over a long period of time are related.

The need for much more laboratory investigation in the purification and evaluation of the various components of the crude mixtures now available is stressed.

I wish to express my thanks to Mr. Cully, of Figge and Hutwelker, New York City, who so kindly supplied an unlimited number of fresh hog kidneys.

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## HISTAMINASE

### RESULTS OF TREATMENT BY INTRAMUSCULAR INJECTION

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IN THE past five years interest has been stimulated in the histaminase treatment of allergic and allied conditions. A considerable literature has thus come into existence. Excellent partial reviews of previous clinical work are to be found in several articles.<sup>1-5</sup> Other papers<sup>6-10</sup> have more complete surveys and extended bibliography. In this presentation, for the sake of brevity, very little reference will be made to other work.

The histaminase solutions used in this study were all prepared from fresh kidney tissue in the laboratory, as described in the paper on laboratory studies.<sup>12</sup> The solution used was the first phosphate buffer extraction of hog kidney powder, varied from light to dark amber in color, and was injected intramuscularly in all cases. Its potency ranged from 5 to 25 units per cubic centimeter (as against histamine phosphate). The usual maximum given at one time was generally 10 c.c. Early in each case injections were usually given twice a week and later, if the case improved, once a week.

The following types and numbers of cases were treated:

|                    |   |
|--------------------|---|
| Asthma             | 7 (2 of these had neurodermatitis)        |
| Neurodermatitis    | 4 (2 of these were of above asthma group) |
| Vasomotor rhinitis | 3   |
| Chronic urticaria  | 2   |
| Serum sickness     | } 1 each                                  |
| Dermatographia     |   |
| Pruritus           |   |
| Migraine           |   |
| Heat allergy       |   |
| Total              | 19 cases                                  |

It is probably best to analyze these cases by tabulations, giving the case identification, duration of symptoms, average dose per week, number of weeks that histaminase was given, length of time of observation from inception of histaminase treatment and outcome. Each tabulation has appended a discussion of special features involved.

Case 5. The first series of injections was given simultaneously with nasal antrum washings. The patient improved rapidly, both as concerned asthma and neurodermatitis. But a relapse occurred despite continued treatment necessitating cessation of injections. During the period of nontherapy she rapidly got much worse. It was difficult to determine whether the histaminase

injections or the antrum washings were responsible for the initial improvement. The second series of injections starting thirteen weeks after the first series was given alone with temporary improvement only.

Case 6. Under treatment there were fewer attacks of dyspnea, but a moderate cough continued. Despite relief the ingestion of interdicted foods would produce slight wheezing.

TABLE I

## ASTHMA

| CASE                                  | DURATION      | DOSE PER WEEK IN UNITS | NO. OF WEEKS  | TIME OF OBSERVATION IN MONTHS | OUTCOME  |
|---------------------------------------|---------------|------------------------|---------------|-------------------------------|--|
| 1. S. G.<br>Simple asthma             | 16 yr.        | 40                     | 10            | 2½                            | Helped at first, worse later                     |
| 2. R. B.<br>Simple asthma             | 5 yr.         | 30                     | 2             | 10                            | Made much worse                                  |
| 3. J. B.<br>Simple asthma             | 2 yr.         | 20                     | 10            | 2½                            | Helped at first, worse later                     |
| 4. L. K.<br>With bronchiectasis       | 5 yr.         | 80                     | 4             | 6                             | No better  |
| 5. E. K.<br>Plus neurodermatitis      | Life          | 70<br>0<br>90          | 10<br>13<br>2 | 5                             | Improved, then worse, etc. <i>See Discussion</i> |
| 6. P. R.<br>Plus infection            | 12 yr.        | 90                     | 12            | 3                             | Better. <i>See Discussion</i>                    |
| 7. A. B.<br>Asthma<br>Neurodermatitis | Life<br>6 mo. |                        |               |                               | <i>See under Case 2, Neurodermatitis group</i>   |

TABLE II

## NEURODERMATITIS

| CASE     | DURATION                       | DOSE PER WEEK IN UNITS | NO. OF WEEKS | TIME OF OBSERVATION IN MONTHS | OUTCOME  |
|----------|--------------------------------|------------------------|--------------|-------------------------------|--|
| 1. E. K. | <i>See Case 5 under Asthma</i> |                        |              |                               |  |
| 2. A. B. | 6 mo.                          | 50<br>100              | 2<br>2       | 6                             | Improved at first, then worse. <i>See Discussion</i> |
| 3. J. B. | 1.5 yr.                        | 556                    | 1            | 2                             | Slight temporary aid. <i>See Discussion</i>          |
| 4. E. L. | Life                           | 900                    | 2            | 2                             | Temporary aid. <i>See Discussion</i>                 |

Case 2. The asthma was controlled very well by routine allergic treatment, but the neurodermatitis persisted. The first injection of histaminase completely relieved the pruritus for a few days, but further injections caused recurrence of asthma and itching. A second series of injections twelve weeks after the first failed to produce any relief.

Case 3. Relief of pruritus was sporadic. The lesions cleared slightly. On release from the hospital there was a severe recurrence.

Case 4. The pruritus was variably relieved. The lesions cleared rapidly. The patient was treated in the hospital and relapsed badly four days after leaving hospital. There was, however, a marked psychic element in this case.

Case 2. The patient developed a tremendous wheal to a preliminary intracutaneous test precluding the use of hog histaminase. A preparation of lamb kidney histaminase having a potency of 1 unit per 3 c.e. was administered.

TABLE III  
VASOMOTOR RHINITIS

| CASE     | DURATION | DOSE PER WEEK IN UNITS | NO. OF WEEKS | TIME OF OBSERVATION IN MONTHS | OUTCOME |
|----------|----------|------------------------|--------------|-------------------------------|---------|
| 1. H. S. | 5 yr.    | 45                     | 4            | 1½                            | No help |
| 2. G. D. | 5 yr.    | 44                     | 6            | 5                             | No help |
| 3. J. D. | 5 yr.    | 71                     | 5            | 5                             | No help |

TABLE IV  
CHRONIC URTICARIA

| CASE               | DURATION | DOSE PER WEEK IN UNITS | NO. OF WEEKS | TIME OF OBSERVATION IN MONTHS | OUTCOME                        |
|--------------------|----------|------------------------|--------------|-------------------------------|--------------------------------|
| 1. N. G.<br>Female | 1 yr.    | 158                    | 2            | 4                             | No help                        |
| 2. N. G.<br>Male   | 3 yr.    | 3 (lamb)               | 11           | 7                             | No help. <i>See Discussion</i> |

Three units were given for eleven weeks without any help. Histamine injections produced a gratifying amelioration of all pruritus. However, the hives continued to appear, although they did not itch. This patient was sensitive to cold as demonstrated by the ice test.

TABLE V  
MISCELLANEOUS

| CASE                    | DURATION | TREATMENT  | TIME OF OBSERVATION IN MONTHS | OUTCOME                             |
|-------------------------|----------|--|-------------------------------|-------------------------------------|
| E. S.<br>Serum sickness | 4 days   | 1 injection of 60 units                          | ½                             | Permanent relief in 3 hours         |
| E. J.<br>Dermatographia | 3 yr.    | 300 units in 2 weeks                             | 2                             | No help                             |
| J. B.<br>Pruritus       | 1 mo.    | 1 injection of 60 units                          | 6                             | Permanent relief in few hours       |
| G. T.<br>Migraine       | 2 yr.    | 2 injections of 75 units each on successive days | 2 days                        | No help                             |
| A. V.<br>Heat allergy   | 5 yr.    | <i>See Discussion</i>                            | 12                            | Helped second time with large doses |

The case of heat allergy (A. V.) was previously described in some preliminary work on histaminase.<sup>11</sup> At that time very small doses of histaminase (less than 1 unit) relieved the swelling of the feet occasioned by hot weather. The following summer nothing was done. The heat of the 1940 summer produced painful ankle edema again. It was now necessary to give about 160 units in two weeks to produce relief. The first injection induced an immediate violent asthmatic attack and a severe local reaction, but subsequent injections elicited only the local reaction in diminishing severity.

#### COMMENT

In all patients a preliminary intracutaneous test for pork sensitivity was performed with the solution. In one patient (2. N.G., chronic urticaria) the

local reaction was so large that lamb histaminase was substituted. In general, a mild or even moderate local reaction is no contraindication to treatment, although the usual care must be exercised. One case (A. V., heat allergy) developed an immediate constitutional reaction in ten minutes. One patient (A. B., asthma-neurodermatitis) had a delayed general effect of asthma in four hours after injection. Neither of these patients developed asthma on subsequent injections. Other general reactions, as reported by others, such as vertigo, nausea, flatulence, laxation, or headache;<sup>7</sup> and fever, malaise, or joint pains<sup>8</sup> were not observed in this series.

Local reactions, varying from mild redness and swelling to those described by Greenbaum,<sup>14</sup> were seen in several cases. The latter consisted of erythematopapular areas at the site of injection, several inches across, with raised margins, marked itching, and local heat. After several inoculations the severity would diminish. There was never any evidence of necrosis. Local pain lasting ten minutes was elicited in all cases immediately upon injection of histaminase solution.

In evaluating any beneficial results one must take into account the well-known tendency of allergic diseases to improve and retrogress spontaneously. Hence, in a small series of cases as presented here, final conclusions cannot be drawn, and only the most meager clues or indications can be found as to the efficacy of this type of medication. For final statistical evaluation in this type of therapy, considering both the unknown quantities in the disease and in the medication, a very large series of cases with adequate controls (both auto- and hetero-) is indicated. In addition, any possible effect of nonspecific protein cannot be minimized. It should be remembered that each cubic centimeter of solution contains approximately 0.9 mg. of protein nitrogen.

Of the six cases treated for asthma, one was considerably improved, three were temporarily relieved, and two were not improved. One of the latter had a well-established bronchiectasis. Commenting, one could say that nonspecific protein might have given similar results.

Of the four cases of neurodermatitis, two (A. B. and E. K.) had variable relief, i.e., they were helped at one time and not at another; and two (J. B. and E. L.) who were hospitalized might have improved just from the hospital stay. Results attained here might have been obtained by other measures, such as calcium intravenously, bed rest, and even suggestion (hypnosis). However, the outstanding feature in all cases of pruritus, of whatever origin, where the medication did help, was the dramatic relief of itching in several hours.

None of the three cases of vasomotor rhinitis had the slightest relief.

Two cases of chronic urticaria had no relief whatsoever. This is in agreement with the much larger series of Piness and Miller,<sup>13</sup> who employed an oral preparation.

One case of serum sickness responded dramatically. This is in accord with the work of Foshay and Hagebusch,<sup>1</sup> although they gave the preparation orally and their injections in much smaller doses.

There was no response in either the one case of dermatographia or in the single case of migraine.

The ankle edema due to heat required a much greater dose the second summer of treatment than the first.

It is somewhat difficult to correlate these results of intramuscular injections with those of other observers who have given the oral preparation, in view of the experiments in the laboratory presentation<sup>12</sup> which demonstrated that histaminase is inactivated by trypsin. Possibly this may explain the advocated necessity of larger doses orally.

As tentative conclusions in this study it might be stated that histaminase by injection, using our present preparations, may prove to be of value in resistant serum sickness, in idiopathic pruritus, and in heat and cold allergy. It may have a limited usefulness in neurodermatitis. It has an unpredictable effect on simple asthma, and no effect on vasomotor rhinitis, dermatographia, migraine, and chronic urticaria. It was not tried in hay fever.

Various lines of evidence, considered logically, lead to the impression that the beneficial effects of histaminase observed by various authors who have used the preparation both orally and by injection, are due to something else in the preparation other than the enzyme-system histaminase. The successful results reported by Altose,<sup>5</sup> Goldberg,<sup>6</sup> Foshay and Hagebusch,<sup>1</sup> Roth and Rynearson,<sup>15</sup> Prickman and co-workers,<sup>16</sup> and Roth and Horton,<sup>17</sup> in its oral administration, despite the fact that histaminase is destroyed by stomach acidity, pepsin, and trypsin, indicate that there is something *other than* the enzyme which is responsible for the improvement. The work of Fox and Harned<sup>18</sup> in which they used a cresolated solution of hog nasal mucosal extract by injection for chronic and acute rhinosinusitis with good results, point in the same direction. Cresol is known to destroy histaminase.<sup>19</sup> The very properties of the enzyme-system histaminase militate against its ability to relieve the ill effects of the theoretical instantaneous release of histamine in anaphylactic shock and allergic reactions. The destruction of histamine by histaminase is not instantaneous and is generally quite slow. It is extremely doubtful that a sudden release of histamine in the body could be counteracted by administration of the enzyme before toxic effects had been produced. Thus, logically one is inclined to believe that any beneficial action reported in any condition for which histaminase was given orally or by injections was due to something else in the protein of the hog kidney or intestinal mucosa.

Finally, much more clinical work must be done with a potent, highly purified histaminase before the validity of any claims as to its efficacy as histaminase alone can be firmly established. Naturally, this involves further laboratory work in the purification and isolation of the enzyme and then the consistent evaluation of its effects in animals. Only when we have done these can we correctly interpret the separate effects of the histaminase and the other protein and nonprotein constituents of the solution.

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## REGIONAL AND SEASONAL VARIATIONS IN THE SERUM CHOLINE-ESTERASE OF HUMAN BEINGS AND DOGS\*

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HALL and Lucas<sup>1</sup> in 1937 reported determinations of serum choline-esterase in 40 normal persons. They found values ranging from 1.32 to 3.61, with an average of 2.50. While determining serum esterase activity in a series of patients with peptic ulcer,<sup>2</sup> we had occasion to check normal values in this region. Determinations were made on 50 normal persons (students and secretaries). In this series were found values varying from 2.94 to 6.58, with an average of 4.72. The great difference between these values and those of Hall and Lucas assumes much importance in the comparison of pathologic cases with normals.

We are unable to offer any satisfactory explanation for the above discrepancy, except that possibility that a climatic or geographic factor is responsible. Without implying that choline-esterase is related to thyroid activity, the fact may be cited that Ring<sup>3</sup> has shown that exposure to temperatures of 0° to 5° C. for periods of three weeks or more brings about a definite increase in thyroid activity in rats. Further, it is generally recognized that the normal standards established for basal metabolic rates in the colder climates are

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\*From the Laboratory of Physiology and Pharmacology, Baylor Medical School, Dallas. Received for publication, January 21, 1941.

approximately 15 per cent above those encountered in the southern states. However, if climatic conditions are responsible for the observed differences in choline-esterase values, it appears that they produce these changes very slowly in that no significant seasonal variations have been observed in our series nor have we been able to demonstrate seasonal variations in serum esterase activity in dogs.

To find whether sudden wide changes in body temperatures alter serum choline-esterase values, we have made determinations of choline-esterase activity in a series of six persons who were being subjected to artificial fever therapy. Blood samples were obtained from the patient immediately before entering the machine and again after five to seven hours during which time a body temperature of 104.6° to 106.6° F. had been attained.\* The procedure revealed no consistent change in esterase values. The average value obtained in 9 determinations before the induction of fever was 4.21, whereas the average value afterward was 4.37.

TABLE I

MEAN WEEKLY CHOLINE-ESTERASE ACTIVITY IN FIVE DOGS FOR A PERIOD OF ONE YEAR

| DATE    | MEAN<br>VALUE | STANDARD<br>DEVIATION | DATE     | MEAN<br>VALUE | STANDARD<br>DEVIATION |
|---------|---------------|-----------------------|----------|---------------|-----------------------|
| 1/18/38 | 1.75          | 0.19                  | 7/29/38  | 1.90          | 0.22                  |
| 1/25/38 | 1.95          | 0.21                  | 8/ 5/38  | 1.72          | 0.18                  |
| 2/ 1/38 | 1.98          | 0.42                  | 8/16/38  | 1.98          | 0.10                  |
| 2/ 7/38 | 1.81          | 0.26                  | 8/20/38  | 1.88          | 0.25                  |
| 2/14/38 | 1.77          | 0.22                  | 8/26/38  | 1.82          | 0.16                  |
| 2/21/38 | 1.57          | 0.14                  | 9/ 2/38  | 2.03          | 0.32                  |
| 2/28/38 | 1.78          | 0.08                  | 9/ 9/38  | 2.00          | 0.26                  |
| 3/ 7/38 | 2.14          | 0.14                  | 9/16/38  | 2.08          | 0.10                  |
| 3/14/38 | 2.16          | 0.30                  | 9/23/38  | 1.77          | 0.23                  |
| 3/21/38 | 2.53          | 0.15                  | 9/30/38  | 1.73          | 0.28                  |
| 3/28/38 | 3.12          | 0.57                  | 10/ 7/38 | 1.68          | 0.13                  |
| 4/ 8/38 | 1.93          | 0.26                  | 10/14/38 | 1.71          | 0.24                  |
| 4/15/38 | 1.77          | 0.32                  | 10/21/38 | 1.75          | 0.09                  |
| 4/22/38 | 2.14          | 0.21                  | 10/28/38 | 1.66          | 0.26                  |
| 4/29/38 | 2.11          | 0.16                  | 11/ 4/38 | 1.86          | 0.16                  |
| 5/ 6/38 | 1.77          | 0.18                  | 11/11/38 | 1.62          | 0.13                  |
| 5/13/38 | 2.08          | 0.23                  | 11/18/38 | 1.65          | 0.05                  |
| 5/20/38 | 2.36          | 0.20                  | 11/25/38 | 1.82          | 0.24                  |
| 5/27/38 | 2.70          | 0.42                  | 12/ 2/38 | 1.79          | 0.17                  |
| 6/ 3/38 | 2.41          | 0.15                  | 12/ 9/38 | 1.88          | 0.16                  |
| 6/10/38 | 2.14          | 0.28                  | 12/16/38 | 1.82          | 0.34                  |
| 6/17/38 | 2.05          | 0.12                  | 12/23/38 | 1.03          | 0.25                  |
| 6/24/38 | 1.82          | 0.25                  | 12/30/38 | 1.95          | 0.19                  |
| 7/ 1/38 | 2.05          | 0.33                  | 1/ 2/39  | 2.05          | 0.08                  |
| 7/ 8/38 | 1.85          | 0.21                  | 1/ 9/39  | 2.00          | 0.36                  |
| 7/15/38 | 1.65          | 0.21                  | 1/16/39  | 1.97          | 0.13                  |
| 7/22/38 | 1.56          | 0.15                  |          |               |                       |

In order to check this wide variation of serum choline-esterase values in human beings, we have made determinations of serum choline-esterase activity at weekly intervals for one year in a series of five dogs. We felt such a comparison would cross check these differences and also bring out any seasonal variations. Analyses were carried out according to the technique described by Hall and Lucas.<sup>1</sup> The values obtained are summarized in Table I in which, to avoid excessive length, is recorded only the mean value for the group of five animals for each date, together with the calculated standard deviation for that date.

\*In one patient two treatments caused an elevation of body temperature to only 101° and 101.4° F.

Daily temperature records reveal that the plateau of the temperature curve extends from April 29 to September 16. The mean esterase value for this interval (twenty-one weeks) is 1.99, with a standard deviation\* of 0.23. The mean value for the remainder of the twelve-month interval is 1.88, with a standard deviation of 0.33. Thus the values are slightly higher during the warmer period, but the difference is insignificant. It is worthy of note that, whereas the mean value for serum esterase activity (1.92) for this series of dogs is less than one-half that found in our series of normal human beings (4.72) in whom determinations were made concurrently, Hall and Lucas,<sup>1</sup> and Hall and Ettinger<sup>4</sup> find no great difference in choline-esterase activity in the two species.

#### SUMMARY AND CONCLUSIONS

1. Normal human serum choline-esterase values in Texas appear to be approximately twice those reported from Toronto, but no consistent seasonal variations have been noted.
2. Elevation of body temperature by artificial fever therapy does not alter human serum choline-esterase values.
3. There is no significant seasonal variation in serum choline-esterase activity in normal dogs.
4. Whereas there appears to be a geographic or climatic difference in serum choline-esterase activity in the human being, values reported here for dogs approximate those reported from Toronto.

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\*Standard deviation is calculated by the formula  $\sigma = \frac{D^2}{(n-1)}$  where  $D^2$  equals the sum of the squares of the difference of each value from the mean value of the group.  $n$  equals number of determinations in the group.



# THE FRACTIONATION OF THE BLOOD IODINE\*

## I. FINDINGS IN PATIENTS WITH NORMAL THYROID FUNCTION AND WITH HYPOTHYROIDISM

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EXTENSIVE investigation has now firmly established the close relation between thyroid function and the quantity of iodine ordinarily circulating within the blood. In the absence of iodine administration in any form the blood iodine is usually decreased in patients with underactive thyroid glands and is increased in those with overactive thyroid glands. The normal range for the iodine content of whole blood varies considerably with the method used for its determination; nevertheless, all methods usually show an increased or decreased blood iodine in hyperthyroidism or hypothyroidism, respectively. These widely reported studies, and particularly the conclusions drawn from them, have formed the groundwork for further insight into the nature of disturbed thyroid function. Moreover, they have disclosed that significant knowledge may be derived from a careful differentiation of the various iodine compounds found in the blood and an interpretation of their possible metabolic significance.

At present, however, the status of both qualitative and quantitative microchemistry is such that the accurate identification and quantitation of these individual iodine-containing substances is hardly practicable. Nevertheless, there is a constant and ever-increasing attempt at the separation and identification of these compounds with an especial effort directed toward the *quantitative determination of the circulating thyroid hormone*.

Various methods have been employed in several attempts to quantitate the circulating thyroid hormone. Barkan<sup>1</sup> attempted to separate the "organic" blood iodine from the "inorganic" by precipitation with silver nitrate. Techniques based on the precipitation of thyroglobulin by a specific antithyroglobulin serum have been applied by other investigators.<sup>2-7</sup> Alcohol has been employed by Perkin and Hurxthal<sup>8</sup> and Trevorrow<sup>11</sup> as a precipitant in order to separate the blood iodine into soluble and insoluble fractions. Other workers<sup>9-11</sup> have employed acetone as the precipitating and thus the fractionating agent. McCleendon and his associates<sup>12-15, 17</sup> employ methanol followed by acetone in their procedure for the fractionation of blood and tissue iodine.

In this report an attempt is made to evaluate the fractionation of the blood iodine in euthyroid persons of this region and in hypothyroid patients. We have employed an acetone procedure. Special attention is paid the insoluble fraction. We presume that this fraction contains the circulating thyroid hormone.

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TABLE I  
EUTHYROID INDIVIDUALS  
(Fractionated Blood Iodine)

| NO. | AGE | SEX | B.M.R.<br>PER CENT | ACETONE<br>AND<br>WATER<br>SOLUBLE<br>I <sub>2</sub> | INSOLU-<br>BLE I <sub>2</sub> | TOTAL I <sub>2</sub> | DIAGNOSIS  |
|-----|-----|-----|--------------------|--|-------------------------------|----------------------|--|
| 1   | 29  | M   | Plus 20            | 3.64   | 0.51                          | 4.15                 | 8 days preoperative for right indirect inguinal hernia |
|     |     |     | Minus 3            | 3.20   | 0.51                          | 3.71                 | 7 days postoperative to right indirect inguinal hernia |
| 2   | 7   | M   | Minus 9            | 3.70   | 0.53                          | 4.23                 | Normal   |
| 3   | 25  | M   | Minus 8            | 5.14   | 1.34                          | 6.48                 | Normal 7/24/39   |
|     | 26  |     | Minus 7            | 3.13   | 0.59                          | 3.72                 | Normal 7/30/40   |
|     | 26  |     | Minus 12           | 2.96   | 0.54                          | 3.50                 | Normal 8/6/40  |
| 4   | 34  | M   | Plus 16            | 3.20   | 1.20                          | 4.40                 | Acromegaly 6/18/40                                     |
|     |     |     | Plus 12            | 4.14   | 0.56                          | 4.70                 | Acromegaly 8/22/40                                     |
| 5   | 33  | F   | Minus 2            | 3.81   | 0.63                          | 4.44                 | Normal   |
| 6   | 23  | F   | Plus 1             | 3.30   | 0.65                          | 3.95                 | "Nervousness"  |
| 7   | 42  | M   | Minus 16           | 2.80   | 0.68                          | 3.48                 | Chronic irritable colon;<br>"nervous exhaustion"       |
| 8   | 55  | F   | -                  | 3.47   | 0.76                          | 4.23                 | Skull fracture   |
| 9   | 46  | M   | Plus 9             | 3.22   | 0.93                          | 4.15                 | Carcinomatosis   |
| 10  | 22  | M   | Minus 5            | 3.56   | 0.94                          | 4.50                 | Hypoparathyroidism                                     |
| 11  | 32  | M   | Minus 10           | 2.62   | 1.01                          | 3.63                 | Normal   |
| 12  | 49  | M   | Plus 16            | 2.96   | 1.02                          | 3.98                 | Chronic ulcerative colitis                             |
| 13  | 44  | F   | Minus 10           | 3.56   | 1.18                          | 4.74                 | 7 days postoperative to herniorrhaphy 12/29/39         |
|     |     |     | Minus 3            | 6.00   | 1.10                          | 7.10                 | 9/19/40  |
| 14  | 24  | M   | Minus 14           | 3.74   | 1.26                          | 5.00                 | Normal   |
| 15  | 25  | M   | Plus 6             | 3.16   | 1.27                          | 4.43                 | Arrested tuberculosis                                  |
| 16  | 52  | M   | Plus 11            | 3.80   | 1.27                          | 5.07                 | "Nervousness"; "anxiety"                               |
| Av. | 33  |     | Plus 1             | 3.58   | 0.88                          | 4.45                 |  |

All iodine values are in micrograms per 100 c.c.

TABLE II  
HYPOTHYROIDISM  
(Fractionated Blood Iodine)

| NO. | AGE | SEX | B.M.R.<br>PER CENT | ACETONE<br>AND<br>WATER<br>SOLUBLE<br>I <sub>2</sub> | INSOLU-<br>BLE I <sub>2</sub> | TOTAL I <sub>2</sub> | DIAGNOSIS                            |
|-----|-----|-----|--------------------|--|-------------------------------|----------------------|--------------------------------------|
| 1   | 52  | M   | Minus 28           | 1.94   | 0.23                          | 2.17                 | Hypothyroidism                       |
| 2   | 33  | M   | Minus 15           | 2.36   | 0.32                          | 2.68                 | Hypothyroidism                       |
| 3   | 38  | F   | 0                  | 2.62   | 0.42                          | 3.04                 | Hypothyroidism; "nervousness"        |
| 4   | 34  | F   | Minus 19           | 3.62   | 0.46                          | 4.08                 | Hypothyroidism                       |
| 5   | 11  | M   | Minus 24           | 3.64   | 0.46                          | 4.10                 | Hypothyroidism                       |
| 6   | 24  | M   | Minus 13           | 3.89   | 0.51                          | 4.40                 | Hypothyroidism                       |
| 7   | 33  | M   | Minus 10           | 2.80   | 0.51                          | 3.31                 | Mild hypothyroidism                  |
| 8   | 45  | M   | Minus 20           | 4.66   | 0.58                          | 5.24                 | Hypothyroidism                       |
| 9   | 26  | M   | Minus 1            | 2.46   | 0.59                          | 3.05                 | Mild hypothyroidism, obesity 8/16/40 |
|     |     |     | Minus 4            | 2.79   | 0.59                          | 3.38                 | Mild hypothyroidism, obesity 11/4/40 |
| 10  | 39  | F   | Minus 24           | 3.38   | 0.59                          | 3.97                 | Mild hypothyroidism                  |
| 11  | 42  | M   | Minus 14           | 3.50   | 0.63                          | 4.12                 | Mild hypothyroidism                  |
| Av. | 35  |     | Minus 14           | 3.14   | 0.49                          | 3.62                 |                                      |

All iodine values are in micrograms per cent.

## METHOD

Fifty milliliters of oxalated blood is pipetted into three volumes of iodine-free absolute acetone. During the addition the mixture is agitated by rotating the flask in order to insure an even and fine precipitation of the blood proteins. The blood-acetone mixture is then allowed to stand at laboratory temperature for twelve hours to insure complete precipitation of the blood proteins and the maximum diffusion of acetone-soluble substances of the blood into the solvent.

The precipitate is then separated from the solution by filtration through a Buchner funnel, using a 11 cm. (589) "Blue Ribbon" Schleicher and Schull filter paper. The precipitate is then washed with 500 ml. of a 75 per cent acetone-water solution. It is further washed with 500 ml. of double-distilled iodine-free water. The filtrate and washings are pooled and analyzed for total iodine content. This is termed the acetone and water *soluble fraction*.

The remaining precipitate containing the acetone and water *insoluble fraction* is removed quantitatively from the funnel and analyzed for total iodine. The Matthews, Curtis, and Brode<sup>16</sup> modification of the Leipert procedure was employed in all our determinations.

## OBSERVATIONS

In 20 determinations made on 16 patients without detectable evidence of thyroid disease, the basal metabolic rate averaged plus 1, ranging from minus 16 to plus 20 (Table I). The whole blood iodine of this group varied from 3.5 to 7.1 micrograms per 100 c.c. averaging 4.45, which is normal for persons in this region on a nonconstant food regimen. The acetone and water *insoluble fraction* averaged 0.88, with variations of from 0.51 to 1.34 micrograms per 100 c.c. The acetone and water *insoluble fraction* does not closely parallel the basal metabolic rate. However, upon analysis of the individual patients with particular reference to the cause of the increased oxygen consumption, a clearer view of the significance of the *insoluble fraction* and its value as a differential diagnostic agent becomes apparent. In Tables I and III it may be noted that persons with increased basal metabolic rates not clinically referable to the thyroid gland present a normal or even lower than normal *insoluble fraction*.

The *insoluble fraction* even fluctuates in the same person (Table I). Moreover, this fluctuation shows only little relationship to the concurrent fluctuations in the basal metabolic rate. Although these individual fluctuations remain within the limits of what we have found to be the normal range for the insoluble blood iodine, they are difficult to comprehend without assuming an unexpectedly wide normal range in the circulating thyroid hormone. All determinations were made on bloods drawn in the morning in the postabsorptive state.

Table II presents a series of determinations made on patients with definite clinical evidence of hypothyroidism. The basal metabolic rate in this group averages minus 14, with variations from 0 to minus 28 per cent. The whole blood-iodine range is from 2.2 to 5.2, with an average of 3.6 micrograms per 100 c.c. The *insoluble fraction* averages 0.49, with variations from 0.23 in a patient with unusually well-developed hypothyroidism to 0.63 microgram per

100 c.c. in a patient with moderate but clinically obvious hypothyroidism. The upper limit of the insoluble blood iodine in this series is thus seen to overlap the lower limit of the insoluble blood iodine of the series of patients with normal thyroid function presented in Table I. (Fig. 1). However, this overlapping of

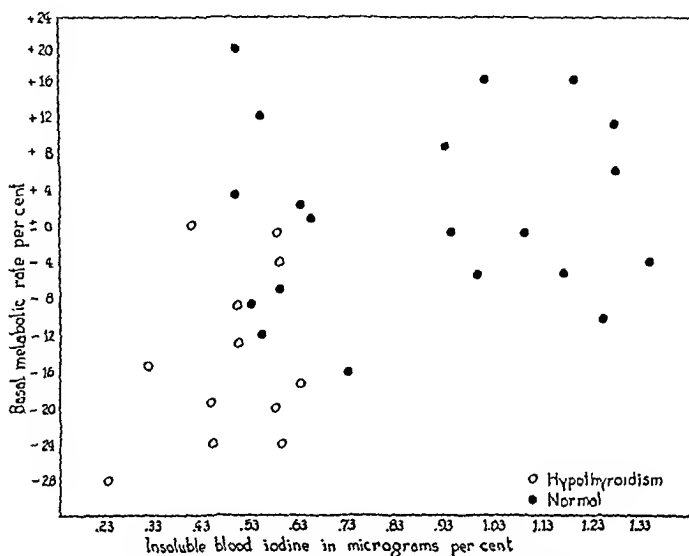


Fig. 1.—A comparison of the ranges of the insoluble blood iodine fraction in patients with normal thyroid function and with hypothyroidism.

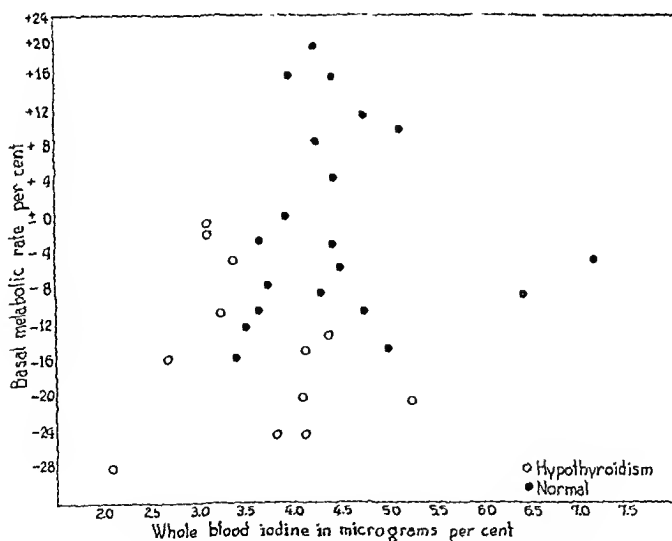


Fig. 2.—A comparison of the ranges of the whole blood iodine in patients with normal thyroid function and with hypothyroidism.

the insoluble blood iodine is not as extensive as that of the total blood iodine of the same two series (Fig. 2). Due to theoretical variation in the sensitivity of individuals to the thyroid hormone, as well as from their variable physiologic requirements for the hormone, a certain amount of overlapping might even be

expected, especially in the borderline cases. *The insoluble blood iodine is consistently low in patients with hypothyroidism.*

Four euthyroid individuals with cardiovascular disease (Table III) showed a range in basal metabolic rate of from plus 9 to plus 46 per cent. Two had a normal blood iodine, and two had a total blood iodine below normal. In all except the one with uncomplicated essential hypertension, the insoluble blood iodine was below the lowest limit of normal. The patient with essential hypertension presented a normal insoluble blood iodine.

TABLE III

PATIENTS WITH DEFINITE CARDIOVASCULAR DISEASE AND WITHOUT CLINICAL EVIDENCE OF THYROID DISEASE

(Fractionated Blood Iodine)

| NO. | AGE | SEX | B.M.R.<br>PER CENT | ACETONE<br>AND<br>WATER<br>SOLU-<br>BLE I <sub>2</sub> | INSOLU-<br>ABLE I <sub>2</sub> | TOTAL I <sub>2</sub> | DIAGNOSIS  |
|-----|-----|-----|--------------------|--|--------------------------------|----------------------|--|
| 1   | 65  | F   | Plus 29            | 2.88   | 0.42                           | 3.30                 | Hypertension; congestive heart failure; (16 months after sub-total thyroidectomy for diffuse hyperplastic goiter with nodules) |
| 2   | 72  | M   | Plus 9             | 2.70   | 0.42                           | 3.12                 | General arteriosclerosis; arteriosclerotic heart disease   |
| 3   | 36  | M   | Plus 46            | 4.40   | 0.44                           | 4.84                 | Hypertensive cardiovascular disease; early congestive failure  |
| 4   | 21  | F   | Plus 24            | 3.63   | 0.71                           | 4.34                 | Essential hypertension without complications   |
| Av. | 48  |     | Plus 27            | 3.40   | 0.50                           | 3.90                 |  |

All iodine values are in micrograms per 100 c.c.

We interpret these findings (Table III), not as hypothyroidism masked by hypertension, but as a subdued thyroid secretion compensatory to the increased supply of oxygen to the tissues as a result of the hypertensive circulation. If the thyroid hormone acts as a catalyst, increasing the utilization of oxygen by the individual tissues, and the tissues are already receiving, through an increased circulation, an abundance of oxygen for their metabolic activity, then the thyroid gland may theoretically, at least, compensate by decreasing the supply of circulating thyroid hormone. Although this series is too small from which to conclude, the consistently low insoluble fraction is apparently of value in differentiating hypertension from hyperthyroidism.

#### SUMMARY AND CONCLUSIONS

A method for the fractionation of the blood iodine into an acetone and water *soluble* fraction and an acetone and water *insoluble* fraction is presented.

The acetone and water *insoluble blood iodine* averages 0.88, with variations of from 0.51 to 1.34 micrograms per 100 c.c. in euthyroid persons without cardiovascular disease.

The acetone and water *insoluble blood iodine* is consistently low in hypothyroidism and ranges from 0.23 to 0.63, with an average of 0.49 microgram per 100 c.c. The *insoluble blood iodine* in hypothyroidism does not show as extensive overlapping into the normal range as does the whole blood iodine.

Hypertensive individuals that are clinically euthyroid present a low normal to lower than normal *insoluble blood iodine*. The low *insoluble* fraction of hypertensive patients may prove to be of diagnostic value in differentiating uncomplicated hypertension from hyperthyroidism. The *insoluble blood iodine* fluctuates individually.

The acetone and water *insoluble blood iodine*, as determined by the method presented, would appear to contain the circulating thyroid hormone.

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## VALUES FOR ACETYLCHOLINE ESTERASE IN THE BLOOD SERUM OF NORMAL PERSONS AND PATIENTS WITH VARIOUS DISEASES\*

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THE presence of an enzyme of the blood capable of hydrolyzing acetylcholine was first suggested by Dale,<sup>1</sup> but not until eighteen years later was its specificity definitely demonstrated by the Stedmans and Easson.<sup>2</sup> Under the leadership of Stedman and his associates rapid progress was made in the study of the physiologic and chemical properties of acetylcholine esterase, but the possible clinical importance of this new enzyme received little attention. Several of the earlier clinical investigators observed wide variation of the esterase activity among various persons on whom studies were carried out over periods of many weeks<sup>3, 4</sup> and for the most part were unable to correlate the acetylcholine esterase activity of the serum with any physiologic or pathologic condition.<sup>5</sup>

Within the past few years, however, several reports have appeared which suggest that the acetylcholine esterase activity of the blood serum may be altered in several clinical syndromes. Antopol and his associates,<sup>6, 7</sup> and McArdle,<sup>8</sup> reported low values for acetylcholine esterase in diseases of the liver and biliary tract, anemia, and hyperpyrexia. Milhorat<sup>5</sup> considered the low values in his group of 109 patients to be the result of debility or severe cachexia, as did Jones and Stadie.<sup>9</sup>

The present study was undertaken to determine if possible the clinical significance of low or high values of acetylcholine esterase in the blood serum of man. The values for acetylcholine esterase were obtained at a constant temperature of 30° C. by utilizing the Cameron glass electrode assembly. Twenty cubic centimeters of the substrate of acetylcholine bromide, containing 2 mg. per cubic centimeter, were placed in a vessel which was surrounded by a continuously flowing current of water at 30° C. The substrate was brought to a pH value of 8.5 by the addition of approximately one drop of tenth-normal solution of sodium hydroxide. To this was added 0.2 c.c. of serum, and with manual stirring, enough hundredth normal solution of sodium hydroxide was added at intervals of five minutes for a period of thirty minutes to maintain the pH value of the substrate at 8.5. A microburette was used for this purpose. A control or blank determination was run from which serum was omitted; the value obtained was subtracted from the value obtained with the serum. Values are expressed as the total cubic centimeters of hundredth normal solution of sodium hydroxide necessary to maintain the substrate at pH 8.5 over a period of thirty minutes when 0.2 c.c. of serum is used.

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## VALUES IN BLOOD SERUM OF NORMAL PERSONS

Values for acetylcholine esterase in the blood serum of 47 normal men and 38 normal women were determined, a single determination for each individual being utilized for this purpose. Among the men the values ranged from 0.91 to 3.13, with an average of  $2.06 \pm 0.07$  and a standard deviation of 0.48, and among the women from 0.90 to 2.99, with an average of  $1.72 \pm 0.08$  and a standard deviation of 0.50. While the variability was essentially the same in the two sexes, the values in the serum of the women were definitely lower than among the men. The average value for the 85 men and women together was 1.90 and the standard deviation was 0.53. If we take a commonly utilized rule of statistics to accept plus or minus twice the standard deviation as the limits of significance (which will include 95 per cent of the cases), we can take  $1.90 \pm 1.06$  or from about 0.85 to 3.0 as acceptable limits of normal variability.

TABLE I

VALUES FOR ACETYLCHOLINE ESTERASE IN A MISCELLANEOUS GROUP OF 24 PATIENTS

| DIAGNOSIS   | ACETYLCHOLINE ESTERASE<br>(c.c.)* |
|---|-----------------------------------|
| Chronic nervous exhaustion  | 1.63                              |
| Erythromelalgia   | 2.45                              |
| Chronic duodenal ulcer with obstruction                           | 0.76                              |
| Acute gouty arthritis and sinus bradycardia                       | 1.95-1.65                         |
| Chronic duodenal ulcer  | 1.38                              |
| Epilepsy with periodic paralysis                                  |                                   |
| Between attacks   | 1.60                              |
| During an attack  | 1.65                              |
| Diabetes mellitus, grade 3  | 1.86                              |
| Obesity   | 2.31                              |
| Urticaria and angioneurotic edema                                 | 2.04-2.20                         |
| Postural hypotension  | 1.00                              |
| Sprue   | 1.64                              |
| Sprue   | 1.77-1.32                         |
| Average in first 12 cases   | 1.70                              |
| Chronic nervous exhaustion  | 1.05                              |
| Chronic nervous exhaustion  | 1.49                              |
| Anxiety neurosis  | 1.02                              |
| Psychoneurosis  | 1.67                              |
| Lipoma of neck  | 1.51                              |
| Indeterminate   | 1.13                              |
| Backache  | 1.72                              |
| Carcinoma of breast with pulmonary metastasis                     | 1.70                              |
| Atopic eczema   | 1.26                              |
| Duodenal ulcer  | 1.73                              |
| Syphilis of the central nervous system; tabes dorsalis; causalgia | 1.55                              |
| Sprue   | 0.92                              |
| Average in second 12 cases  | 1.40                              |

\*Cubic centimeters of 0.01 normal solution of sodium hydroxide.

*Fluctuations in Values for Acetylcholine Esterase in Same Person.*—The results given thus far are for one determination made for each of a number of individuals. The variation, therefore, represents the interindividual variability. In addition to this it was thought desirable to obtain some information concerning the variation of different determinations made for the same individual, that is, the intra-individual variation. Daily estimations were made on the serum of two normal persons. For one, six successive daily determinations had a mean of  $0.86 \pm 0.02$  and a standard deviation of 0.06. For the other, seven



determinations had a mean of  $1.17 \pm 0.05$  and a standard deviation of 0.14. The standard deviation measuring the intra-individual variability judged by these two persons together was 0.11. Thus it is seen that the intra-individual variability is about a fifth of the interindividual variability.

#### VALUES ASSOCIATED WITH VARIOUS DISEASES

*Miscellaneous Group of Cases.*—Values for acetylcholine esterase were determined in 24 patients in whom a variety of conditions was present. These included chronic nervous exhaustion, neurosis, epilepsy, obesity, urticaria, angioneurotic edema, duodenal ulcer, diabetes mellitus, syphilis of the central nervous system, sprue, postural hypotension, and arthritis (Table I). The values with one exception were within the range of normality (0.85 to 3.00), and the exception is not significant.

*Hypertension.*—Determinations of acetylcholine esterase were made in 13 patients with hypertension. The hypertension was of all grades of severity. The values ranged from 1.17 to 2.51 and averaged 1.58. Hypertension apparently does not produce changes in values for acetylcholine esterase.

*Cardiac Disorders.*—Values for acetylcholine esterase were determined in 12 patients with cardiac disorder. Our interest in this group was prompted by the possibility that the acetylcholine esterase content of the serum might be an index of the sympathetic-parasympathetic balance in cases of cardiac irritability, especially in cases of paroxysmal tachycardia of various types.

In a general way it can be stated that the values for this group fall within the limits of variation of the normal. This was found to be true in cases associated with organic cardiac lesions as well as in those in which periodic ectopic rhythms were the only cardiac abnormality. The values can be summarized as follows: 1.80 for a man 32 years old who had mitral stenosis and paroxysmal tachycardia (probably auricular fibrillation); 1.73 for a man 52 years old who had chronic auricular fibrillation associated with arteriosclerotic cardiac disease; 1.88 and 1.57, respectively, for two patients who recently had suffered from attacks of acute coronary thrombosis; 1.78 and 1.79, respectively, for two patients both aged 33 years who had minimal mitral endocarditis associated with considerable anxiety and heart consciousness. Six patients had paroxysmal tachycardia, but no demonstrable evidence of organic heart disease. The values of acetylcholine esterase in these patients were 2.14, 1.89, 1.36, 2.03, 2.31, and 3.31, respectively; all were determined while the cardiac rhythm was normal. Although the value in one was somewhat more than 3.00, which is the upper value of normality, only one such case was found among the 6 studied, and is not considered significant. In the first two cases the opportunity to determine levels of acetylcholine esterase during paroxysmal tachycardia also presented itself; readings were 2.00 and 1.39, respectively (as compared to the levels of 2.14 and 1.89, respectively, when the cardiac rhythm was normal). It is doubtful whether the variations noted are significantly great, but the change was at least in the same direction and the values were lower in the course of paroxysmal tachycardia than in the periods of normal rhythm. Before any definite conclusions could be drawn, however, the concentration of

acetylcholine esterase in serum would have to be determined at more frequent intervals before, during, and after paroxysmal tachycardia.

*Pregnancy.*—The values for acetylcholine esterase in the serum of 14 women, who either were pregnant at the time or had recently given birth to a child, were determined, and the results are listed in Table II. In two women readings were obtained before and after delivery. It will be noted that while the readings obtained in the course of pregnancy fall within the limits of variation of normal, with two exceptions they are all below the mean found for females (1.72). The mean in the 14 cases of pregnancy was  $(1.27 \pm 0.08)$ , and the difference from the mean for females is significant ( $0.45 \pm 0.11$ ). In one instance in which the occasion presented itself to repeat the determination of the value for acetylcholine esterase in the serum five months post partum, there was a considerable increase over the value obtained during the earlier months of pregnancy (3.02 as compared with 1.31). This finding in association with the rather low values noted during pregnancy prompts further investigation of the behavior of acetylcholine esterase in the serum in relation to pregnancy. Opportunity has not appeared thus far to determine values of acetylcholine esterase in the serum in cases of eclampsia.

TABLE II

VALUES FOR ACETYLCHOLINE ESTERASE DURING AND AFTER TERMINATION OF PREGNANCY

| AGE OF PATIENT<br>(YR.) | ANTE PARTUM                          |                       | POST PARTUM                          |                        |
|-------------------------|--------------------------------------|-----------------------|--------------------------------------|------------------------|
|                         | ACETYLCHOLINE<br>ESTERASE<br>(C.C.)* | MONTH OF<br>PREGNANCY | ACETYLCHOLINE<br>ESTERASE<br>(C.C.)* | TIME AFTER<br>DELIVERY |
| 27                      | 1.00                                 | 3                     |                                      |                        |
| 26                      | 1.37                                 | 3                     |                                      |                        |
| 28                      | 1.26                                 | 5                     |                                      |                        |
| 34                      | 1.24                                 | 3                     |                                      |                        |
| 22                      | 0.96                                 | 4                     |                                      |                        |
| 21                      | 1.11                                 | 5                     |                                      |                        |
| 33                      | 1.30                                 | 7                     |                                      |                        |
| 21                      | 1.24                                 | 7                     |                                      |                        |
| 25                      | 0.77                                 | 6                     |                                      |                        |
| 32                      | 1.74                                 | 8                     |                                      |                        |
| 27                      |                                      |                       | 0.75                                 | 2 days                 |
| 17†                     | 1.06                                 | 5                     |                                      |                        |
| 18†                     | 2.01                                 | 8½                    |                                      |                        |
| 29                      | 1.31                                 | 3                     | 3.02                                 | 5 mo.                  |
| 26                      | 1.39                                 | 1 day<br>ante partum  | 1.51                                 | 1 day                  |

\*Cubic centimeter of 0.01 normal solution of sodium hydroxide.

†Two different pregnancies in same case.

*Disease of Liver and Biliary Tract.*—The values in 3 patients with catarrhal jaundice varied from 1.31 to 1.90. The values in 3 patients with stricture of the common bile duct and biliary cirrhosis were low; they were 1.03, 0.74, and 0.92. The values in 2 patients with neoplastic disease of the liver also were low, that is, 1.37 and 0.63. The values in 7 patients with marked hepatitis (surgical description) were low; they were 1.05, 0.69, 1.12, 1.54, 1.01, 0.62, and 0.97, and averaged 1.00. The two values of 0.62 and 0.69 were less than the lower limit of the range of normality. The values in 5 patients with compensated cirrhosis of the liver were just as low as in the group of cases of hepatitis; they were 0.82, 0.89, 0.99, 1.04, and 1.14, and averaged 0.98. It is to be noted

that the values were in the lowest range of values for normal persons, and in one case was slightly less than any of the normal values. The values in 7 patients with decompensated atrophic cirrhosis of the liver were very low; they were 0.22, 0.53, 0.50, 0.62, 0.46, 0.41, and 0.41, and averaged 0.43. In every case the values were much lower than the lowest values for normal persons and much lower than those encountered in severe hepatitis, biliary cirrhosis, and compensated cirrhosis. It is clear that the more extensive and severe the hepatic damage, the lower the values for acetylcholine esterase.

*Pancreatic Disease.*—The values for acetylcholine esterase were 1.46, 1.49, and 1.57 respectively, in 3 patients with subacute pancreatitis, and 1.23, 0.95, 0.18, 1.68, and 1.55 in 5 patients with chronic pancreatitis with recurring exacerbations. The values with the exception of one were within the range found in normal persons; in the patient in whom the value was 0.18, extensive pancreatic damage without recognizable hepatic disease was found at surgical exploration; as the condition of the patient improved, however, the value for acetylcholine esterase gradually increased to 1.03 fourteen days later. This one low value suggests that pancreatic disease may depress values for acetylcholine esterase, but no conclusions can be drawn.

The values for acetylcholine esterase in 8 patients with carcinoma of the head, and one of the body of the pancreas ranged from 0.77 to 1.47, and averaged 1.18. Only one value was below the normal range; in this patient there was extensive hepatic metastasis. In another patient the initial value was 1.43, but in ten days, as the condition became much worse, the value decreased to 0.61. When the degree of hepatic damage is considered in these cases, it is interesting that the values for acetylcholine esterase were as high as they were.

#### EFFECT OF VARIOUS DRUGS ON VALUES FOR ACETYLCHOLINE ESTERASE IN THE SERUM

While no systematic attempt was made to study the effect of various drugs on values for acetylcholine esterase, certain observations were recorded. Values were determined in a case of exophthalmic goiter and myasthenia gravis before the administration of 15 mg. of prostigmine orally and fifteen minutes after administration. The values decreased from 1.57 to 1.15. Values before and after two weeks of histaminase therapy in a patient with erythromelalgia were 2.45 and 2.55, respectively. Values before and ten minutes after intravenous administration of 50 mg. of thiamine chloride were 1.55 and 1.59, respectively. Values before and at the height of the pancreatic response to the administration of secretin intravenously were 1.73 and 1.69, respectively. Histaminase, thiamine chloride, and secretin had no effect in these cases on the values for acetylcholine esterase. On the other hand, prostigmine apparently decreased the values transiently.

#### VALUES IN PANCREATIC JUICE

The values for acetylcholine esterase in pancreatic juice obtained by duodenal tube were 0.14 and 0.0, respectively, ten and twenty minutes after stimulation with purified secretin, and that in pancreatic juice obtained from a human being, who had an external pancreatic fistula, was 0.03.

## COMMENT

Our limited group of cases does not permit an elaborate statistical study, but from these data it seems safe to assume that in the presence of severe hepatic damage the acetylcholine esterase activity of the blood serum is decreased. We have observed that not all diseases of the liver or biliary tract are associated necessarily with a low value of acetylcholine esterase, but rather that the low value of acetylcholine esterase is usually a rough index of the amount of damage to the hepatic parenchyma. These findings are in close agreement with the results of McArdle.<sup>8</sup> In 79 per cent of his 71 cases of disease of the liver, acetylcholine esterase values were below the range for normal adults. In 23 cases of cirrhosis of the liver the mean value was less than half of that of normal adults. There seems to be no satisfactory explanation for the decrease of acetylcholine esterase activity in disease of the liver. It has been suggested that bile salts *in vitro* will inhibit esterase activity,<sup>7, 10</sup> but McArdle's work does not support this hypothesis.

A reduction in values during pregnancy also may be assumed, but a suitable explanation has not yet been advanced.

Our observations in man do not coincide with the observation of Ginsberg and his associates,<sup>11</sup> who found the pancreatic juice of a dog a rich source of acetylcholine esterase. Nor did the parenteral administration of 50 mg. of thiamine chloride to man have any effect on the esterase activity, although an inhibiting effect has been noted in the serum of horses and rats.<sup>12</sup>

## SUMMARY

The values of acetylcholine esterase in the blood serum of normal persons and persons with various diseases were determined. For 47 normal men the mean value was  $2.06 \pm 0.07$  and the standard deviation was 0.48. For 38 normal women the mean was  $1.72 \pm 0.08$  and the standard deviation was 0.50. For all the normal persons taken together, the mean was 1.90 and the standard deviation was 0.53. Accepting  $\pm 2$  times the standard deviation as a reasonable range for normality, we set the range for normal persons as from 0.85 to 3.0.

Values for acetylcholine esterase in the blood serum were within the range of normal in patients with chronic nervous exhaustion, neurosis, diabetes mellitus, epilepsy, obesity, urticaria, angioneurotic edema, duodenal ulcer, syphilis of the central nervous system, sprue, postural hypotension, arthritis, hypertension of all degrees of severity, heart disease, both organic and that in which periodic ectopic rhythms were the only abnormality, and disease of the biliary tract without severe disease of the liver. Values for acetylcholine esterase were significantly low in pregnancy and in instances of severe hepatic damage. A loose parallelism existed between the values for acetylcholine esterase in the serum and the severity of liver disease. The lowest values were obtained in those patients in whom damage to the liver was most severe (decompensated portal cirrhosis).

Values for acetylcholine esterase in patients with pancreatic disease were, with one exception, within the range of normal. The single low value was so low that the possibility of disease of the pancreas lowering values must be entertained.

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# LABORATORY METHODS

## GENERAL

### A "TRANSPARENT CHOCOLATE AGAR" FOR THE PRIMARY ISOLATION OF THE NEISSERIA AND HEMOGLOBINOPHILES\*

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IT IS customary for the personnel of a bacteriologic laboratory to have available, in addition to blood agar, special media for the cultivation of such fastidious organisms as the gonococcus, meningococcus, and influenza bacillus. Many laboratories often employ different media for the isolation of each of the microorganisms mentioned. This imposes a considerable burden on the personnel and on the facilities of the laboratory.

Ever since Cohen and Fitzgerald<sup>1</sup> introduced a cooked blood agar (chocolate agar) and emphasized its usefulness for isolating *Hemophilus influenzae*, many investigators have employed such a medium for the cultivation of both the *Neisseria* and the hemoglobinophiles. McLeod and his co-workers,<sup>2</sup> Leahy and Carpenter,<sup>3</sup> Reitzel and Kohl<sup>4</sup> reported satisfactory results with various "chocolate agars" for the primary isolation of the gonococcus. Janeway,<sup>5</sup> commenting on medical progress in bacteriology, recommends fresh "chocolate agar" plates or slants for the cultivation of the influenza bacillus and meningococcus from spinal fluid.

Recently, bacto proteose No. 3 agar (Difco<sup>6</sup>), enriched with either bacto hemoglobin, 5 per cent heated defibrinated or citrated blood, has been recommended for the cultivation of the *Neisseria*. The medium has been used successfully by Carpenter,<sup>7</sup> and is highly recommended by Herrold,<sup>8</sup> and Sulkin and Gottlieb.<sup>9</sup>

While the efficiency of the proteose No. 3 "chocolate agar" has been established, its opacity presents a disadvantage. Leahy and Carpenter,<sup>3</sup> referring to the cultivation of the gonococcus, are of the opinion that the direct inspection of a "chocolate agar" plate is almost valueless when obscured by a luxuriant growth of other organisms. While the application of the oxidase reaction<sup>2</sup> decreases the difficulty due to the opacity of the medium, the promiscuous use of this test has been shown by Cohn and Kruger<sup>10</sup> to have certain disadvantages. The isolation of small numbers of influenza bacilli, however, from a "chocolate agar" plate presents greater difficulty than is encountered in the isolation of the gonococcus and meningococcus, since no reaction similar to the oxidase test is available.

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Proteose No. 3 "chocolate agar" possesses the qualities of an ideal medium, namely: stability of ingredients, a high degree of efficiency, and simplicity of composition and preparation. A medium of similar composition which would maintain all the above qualities and also be transparent seems very desirable.

It is conventional in the preparation of a "chocolate agar" to add 5 or 10 c.c. of sterile defibrinated or citrated blood to an agar base at 80° to 100° C., followed by pouring the so-called "chocolatized" ingredients into a Petri dish. It was found that by centrifuging the heated, coagulated blood, proteose No. 3 agar combination at a moderate speed, a supernatant fluid resulted which when poured into Petri dishes was transparent. The opaque sediment which is discarded never exceeds 2 c.c. for each 21 c.c. of the centrifuged mixture. The proteose No. 3 agar base was further enriched by the addition of a small amount of soluble starch (0.1 per cent) which aided in the production of larger colonies without overgrowth by other organisms.

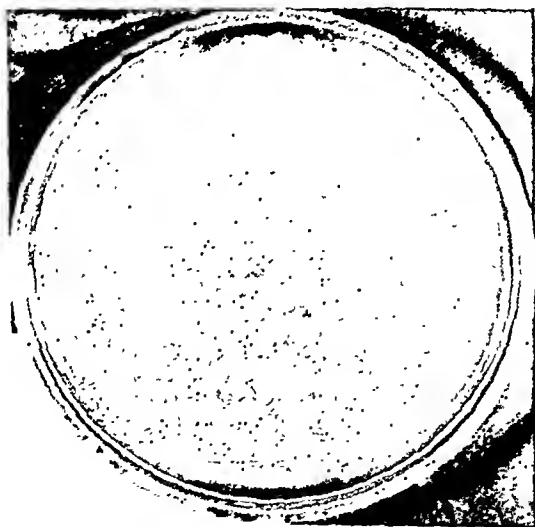


Fig. 1.—Eighteen-hour, primary growth of *Hemophilus influenzae* on "transparent chocolate agar."

The medium in its final form was found to possess all the previously enumerated suitable qualities. Its simplicity of preparation and its ability to support luxuriant primary growth of the meningococcus, gonococcus, and influenza bacillus make it a very suitable medium for the routine of a clinical bacteriologic laboratory. In addition, its transparency presents an added advantage, for the colonies sought can be readily distinguished in mixed cultures.

Over a period of several months we have found this medium a satisfactory addition to the laboratory for the following multiple uses:

1. Routine cultures of spinal fluid and blood from cases of influenzal and epidemic meningitis.
2. Determination of the presence of meningococcus carriers, culturing the material from the nasopharynx.
3. Routine cultures of exudate from all types of gonococcal infections.

4. The isolation of the influenza bacillus from the nose, throat, and sputum.
  5. Isolation of *Hemophilus conjunctivitis* from cases of conjunctivitis.
- We hope to analyze the results of this work in a future publication.

#### PREPARATION

*Stock agar base.* Suspend 1 Gm. of soluble starch in 1,000 c.c. of cold distilled water. Mix well. Add 45 Gm. of proteose No. 3 agar (Difeo), and mix. Boil for one to two minutes to dissolve the agar and starch. Distribute to test tubes in 20 or 40 c.c. amounts, or as desired. Sterilize in autoclave for twenty minutes at 15 pounds pressure.



Fig. 2.



Fig. 3.

Fig. 2.—Eighteen-hour, aerobic, primary growth of the meningococcus.

Fig. 3.—Thirty-six-hour primary growth of the gonococcus in 10 per cent carbon dioxide atmosphere (oxidase positive).

*Preparation of final medium.* For every 20 c.c. of melted stock agar base, which has been cooled to about 50° C., add 1 c.c. of sterile defibrinated or citrated blood (human blood is used in our laboratory). Mix well. Heat mixture in water bath to 80° C. to 85° C. and allow to remain at the temperature until the blood begins to coagulate.

Centrifuge at 2,000 r.p.m. for one to two minutes.

Pour the supernatant fluid into a Petri dish or prepare slants.



## DESCRIPTION OF COLONIES

In eighteen to twenty-four hours colonies of the influenza bacillus are colorless, transparent, and vary in diameter from 1 to 3 mm. On slants growth is luxuriant in eighteen hours, some strains producing colonies which are 2 to 3 mm. in diameter (Fig. 1).

In eighteen to twenty-four hours meningococci appear as circular, moist, shiny colonies, which are colorless by transmitted light. By reflected light the colonies are grayish and semiopaque. The size of the colonies is usually from 2 to 4 mm. in diameter (Fig. 2).

In twenty-four to thirty-six hours colonies of the gonococcus are semiopaque, colorless, opalescent, and have undulated margins. An overcrowded plate may reveal colonies which are 0.5 to 1 mm. in diameter. On a plate which allows ample growing space the colonies may reach 3 mm. in diameter (Fig. 3).

## CONCLUSIONS

1. A modification of "chocolate agar," which is stable, simple to prepare, and yields a transparent medium, is presented.

2. Its ability to support initial growth of the influenza bacillus, meningococcus, and gonococcus make this medium a valuable addition to the clinical bacteriologic laboratory.

3. It eliminates the need for several separate media for the cultivation of the *Neisseria* and the hemoglobinophiles.

We wish to express our appreciation to Dr. Alfred Angrist, pathologist of Queens General Hospital, for his generous advice and cooperation.

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## TITRATION AND STANDARDIZATION OF ANTIGEN FOR THE EAGLE FLOCCULATION TEST\*

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ANTIGENS manufactured in this laboratory for the Eagle flocculation test have repeatedly failed to equal in reactivity standard antigen received from the test originator. Since a method for antigen adjustment has not been available, only two alternatives were open. Many antigen extracts could be made with the hope of finding one of suitable reactivity or a substandard antigen might be used in the test. Neither of these choices seemed to present an acceptable solution, and, for this reason, an investigation of the methods that might be used for the adjustment of this antigen was undertaken.

It is generally agreed that the reactivity of an antigen is influenced by changes in the sterol-lipoid ratio. Since the Eagle antigen closely approaches saturation with sterols, the possibility of increasing this component is limited. In a preliminary study, however, it has been found that an increase in the lipid concentration approximating 100 per cent is possible without necessitating a reduction in the specified sterol concentration. It was also shown that, although some antigen preparations could be adjusted to a satisfactory level through an increase in lipid concentration, others could not be rendered satisfactory by this procedure alone.

Eagle<sup>1</sup> recognized that the electrolyte concentration of the saline diluent may also influence the reactivity level of the antigen, but implied that too great a concentration may have deleterious effects. The results obtained by the combined use of these methods are presented.

Four antigens were prepared from commercial beef heart powder in exact accordance with the published technique of the originator.<sup>2</sup> Sterolization was carried out with Pfanstiehl cholesterol and Difeo corn germ sterol. Dilutions of each of these antigens in 0.85 per cent saline (1 part antigen with 2 parts saline) were prepared and stored in the refrigerator for twenty-four hours. For control purposes a similar antigen dilution was prepared from standard antigen that had been obtained from the originator. These stock antigen dilutions were then rediluted with eight parts of 4 per cent saline and tested with serial serum dilutions.

Serum dilutions for the titration of antigens were prepared by diluting previously tested, pooled, positive sera in negative serum, care being exercised to insure adequate mixing. The following dilution schedule was used:

|         |                  |        |                              |
|---------|------------------|--------|------------------------------|
| Tube 1. | Positive serum - | 3 c.c. |                              |
| Tube 2. | Positive serum - | 2 c.c. | Plus negative serum - 2 c.c. |

\*From the Venereal Disease Research Laboratory, United States Marine Hospital, Staten Island.

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|          |                     |          |                       |          |
|----------|---------------------|----------|-----------------------|----------|
| Tube 3.  | Positive serum -    | 1 c.c.   | Plus negative serum - | 3 c.c.   |
| Tube 4.  | Positive serum -    | 0.5 c.c. | Plus negative serum - | 3.5 c.c. |
| Tube 5.  | Serum from tube 2 - | 0.5 c.c. | Plus negative serum - | 3.5 c.c. |
| Tube 6.  | Serum from tube 3 - | 0.5 c.c. | Plus negative serum - | 3.5 c.c. |
| Tube 7.  | Serum from tube 4 - | 0.5 c.c. | Plus negative serum - | 3.5 c.c. |
| Tube 8.  | Serum from tube 5 - | 0.5 c.c. | Plus negative serum - | 3.5 c.c. |
| Tube 9.  | Negative serum -    | 3 c.c.   |                       |          |
| Tube 10. | Negative serum -    | 3 c.c.   |                       |          |

The results of the preliminary titration of these four antigens and the control are shown in Table I. As indicated in this table the control or standard antigen gave some degree of positivity in the seventh dilution, whereas the trial preparations gave negative macroscopic and microscopic readings considerably above this level. On this basis the trial antigens were not considered as having standard reactivity.

TABLE I

| TUBE | SERUM<br>(0.2 c.c.) | 0.1 C.C. OF ANTIGEN DILUTION<br>(1 PART STOCK ANTIGEN DILUTED + 8 PARTS 4.0% SALINE) |              |              |              |                     |
|------|---------------------|--|--------------|--------------|--------------|---------------------|
|      |                     | ANTIGEN<br>A   | ANTIGEN<br>B | ANTIGEN<br>C | ANTIGEN<br>D | STANDARD<br>ANTIGEN |
|      |                     |  |              |              |              |                     |
| 1    | Positive            | POS.-pos.  | POS.-pos.    | POS.-pos.    | POS.-pos.    | POS.-pos.           |
| 2    | Pos. + Neg. (1:2)   | POS.-pos.  | POS.-pos.    | POS.-pos.    | POS.-pos.    | POS.-pos.           |
| 3    | Pos. + Neg. (1:4)   | POS.-pos.  | POS.-pos.    | POS.-pos.    | POS.-pos.    | POS.-pos.           |
| 4    | Pos. + Neg. (1:8)   | DBT.-pos.  | POS.-pos.    | NEG.-dbt.    | DBT.-pos.    | POS.-pos.           |
| 5    | Pos. + Neg. (1:16)  | NEG.-neg.  | NEG.-dbt.    | NEG.-neg.    | NEG.-neg.    | POS.-pos.           |
| 6    | Pos. + Neg. (1:32)  | NEG.-neg.  | NEG.-neg.    | NEG.-neg.    | NEG.-neg.    | DBT.-pos.           |
| 7    | Pos. + Neg. (1:64)  | NEG.-neg.  | NEG.-neg.    | NEG.-neg.    | NEG.-neg.    | NEG.-dbt.           |
| 8    | Pos. + Neg. (1:128) | NEG.-neg.  | NEG.-neg.    | NEG.-neg.    | NEG.-neg.    | NEG.-neg.           |
| 9    | Negative            | NEG.-neg.  | NEG.-neg.    | NEG.-neg.    | NEG.-neg.    | NEG.-neg.           |
| 10   | Negative            | NEG.-neg.  | NEG.-neg.    | NEG.-neg.    | NEG.-neg.    | NEG.-neg.           |

All tubes shaken for five minutes on Kahn shaking machine and centrifuged at 2,000 r.p.m. before reading.

POS., DBT., and NEG.—Positive, doubtful, and negative *macroscopic* readings.

Pos., dbt., and neg.—Positive, doubtful, and negative *microscopic* readings.

In an effort to adjust these four antigens to the level of reactivity of the control, the method of increasing lipid content was utilized. This was accomplished by evaporating alcohol from measured portions of the unsterolized antigens until reductions to three-fourth and one-half the original volumes were obtained. Thus, when 100 ml. of antigen were reduced to 75 ml., a 33 per cent increase in lipid content was obtained, and when further reduction to 50 ml. was accomplished, a 100 per cent increase in lipid content was effected. These concentrated antigens were sterolized and filtered, and stock antigen dilutions were prepared as previously described. After twenty-four hours' refrigeration portions of each of these stock antigen dilutions were rediluted with eight volumes of 4 per cent, 6 per cent, 8 per cent, and 10 per cent salines, and retitrated against standard antigen using the previously described serum dilutions. To eliminate duplication of data only one protocol, considered to be representative of the group, is presented in Table II.

The findings indicate that antigen A was not adjusted to standard reactivity by the use of increased saline concentrations alone. Nor did increased lipid contents of 33 per cent, or 100 per cent, attain a satisfactory reactivity level when used with 4 per cent saline. However, a reactivity as sensitive as that of the standard antigen was obtained at two points: 33 per cent increased lipid with 10 per cent saline and 100 per cent increased lipid with 8 per cent

saline. Antigen with 33 per cent increased lipid when used with 10 per cent saline, although sufficiently reactive with positive sera, gave unsatisfactory results (see Discussion) in the negative serum tests, so the final adjustment chosen for this antigen was 100 per cent increase of lipoids with 8 per cent saline as the secondary diluent. Similar titrations of the other three antigens, designated 100 per cent increase in lipoids with 8 per cent saline, in one instance, and 100 per cent increase in lipoids with 6 per cent saline, in the other two, as the proper adjustment end points. All four of these antigens, after being adjusted in accordance with the titration results, were found to be of standard reactivity as compared against standard antigen when tested with positive and negative sera.

TABLE II

| TUBE | SERUM                  | ANTIGEN A                           |     |     |      | ANTIGEN A<br>LIPOID CONTENT<br>INCREASED 33% |     |     |      | ANTIGEN A<br>LIPOID CONTENT<br>INCREASED 100% |     |     |     | STAND-<br>ARD<br>ANTI-<br>GEN |    |
|------|------------------------|-------------------------------------|-----|-----|------|--|-----|-----|------|---|-----|-----|-----|-------------------------------|----|
|      |                        | SALINE CONTENT OF SECONDARY DILUENT |     |     |      |  |     |     |      |   |     |     |     |                               |    |
|      |                        | 4%                                  | 6%  | 8%  | 10%  | 4%   | 6%  | 8%  | 10%  | 4%  | 6%  | 8%  | 10% |                               | 4% |
| 1    | Positive               | P-p                                 | P-p | P-p | P-p  | P-p  | P-p | P-p | P-p  | P-p   | P-p | P-p | P-p | P-p                           |    |
| 2    | Pos. + Neg.<br>(1:2)   | P-p                                 | P-p | P-p | P-p  | P-p  | P-p | P-p | P-p  | P-p   | P-p | P-p | P-p | P-p                           |    |
| 3    | Pos. + Neg.<br>(1:4)   | P-p                                 | P-p | P-p | P-p  | P-p  | P-p | P-p | P-p  | P-p   | P-p | P-p | P-p | P-p                           |    |
| 4    | Pos. + Neg.<br>(1:8)   | D-p                                 | P-p | P-p | P-p  | P-p  | P-p | P-p | P-p  | P-p   | P-p | P-p | P-p | P-p                           |    |
| 5    | Pos. + Neg.<br>(1:16)  | N-n                                 | N-n | D-p | D-p  | N-d  | P-p | P-p | P-p  | P-d   | P-p | P-p | P-p | P-p                           |    |
| 6    | Pos. + Neg.<br>(1:32)  | N-n                                 | N-n | N-n | N-d  | N-n  | N-d | N-p | D-p  | N-d   | D-p | D-p | P-p | D-p                           |    |
| 7    | Pos. + Neg.<br>(1:64)  | N-n                                 | N-n | N-n | N*-d | N-n  | N-n | N-n | N*-d | N-n   | N-n | N-d | P-p | N-d                           |    |
| 8    | Pos. + Neg.<br>(1:128) | N-n                                 | N-n | N-n | N*-d | N-n  | N-n | N-n | N*-d | N-n   | N-n | N-n | N-d | N-n                           |    |
| 9    | Negative               | N-n                                 | N-n | N-n | N*-d | N-n  | N-n | N-n | N*-d | N-n   | N-n | N-n | N-n | N-n                           |    |
| 10   | Negative               | N-n                                 | N-n | N-n | N*-d | N-n  | N-n | N-n | N*-d | N-n   | N-n | N-n | N-n | N-n                           |    |

All tubes shaken five minutes on Kahn shaking machine and centrifuged at 2,000 r.p.m. before reading.

Either 0.4 c.c. of serum plus 0.2 c.c. of antigen dilution, or 0.2 c.c. of serum with 0.1 c.c. of antigen dilution may be used.

P, D, N—Positive, doubtful, and negative macroscopic readings.

p, d, n—Positive, doubtful, and negative microscopic readings.

\*Swirl appearance missing.

#### DISCUSSION

The titration results presented in Table II show that the unadjusted antigen and the antigen containing 33 per cent increased lipoids gave rough microscopic pictures with negative sera when the 10 per cent saline diluent was employed. In these tubes the typical negative swirl was missing, the gross appearance being more chalky than usual. For this reason, these adjustment end points could not be considered suitable, even though a satisfactory reactivity with positive serum was attained.

These microscopic readings are considered an essential part of the titration, since they allow acute differential observations to be made. This is especially necessary in the negative serum tests so that an antigen adjustment can be made that will not give rough negative microscopic pictures. However, if two or more

adjustments are found to be equally good, the lesser lipid content with the lower saline concentration is probably to be preferred.

The level of reactivity that is now considered satisfactory for any test antigen is chosen by the author of that particular test. If, for some reason, the test author decides to raise or lower the test sensitivity level by changing the antigen reactivity, readjustments of antigen and saline can be made that will reproduce the desired result. For this reason, it is necessary that a standard antigen be available for comparative antigen titrations.

However, since the two antigen adjustments used are quantitative in nature, it is not presumed that sufficient correction can be obtained by these methods alone to compensate for any qualitative changes that may occur in some beef heart powder. Such unsatisfactory beef heart powder has not been encountered in this laboratory to date.

#### SUMMARY

Four Eagle flocculation antigens prepared from commercial beef heart powders were found to have varied reactivity, none of which were equal in reactivity to standard antigen obtained from Eagle.

A titration method for the comparison of antigen reactivity and a procedure which will allow the accurate adjustment of Eagle flocculation antigen is described.

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## A STUDY OF RECENTLY ISOLATED STRAINS OF STAPHYLOCOCCI AND THEIR ABILITY TO COAGULATE HUMAN PLASMA\*

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MOST of the literature published the last few years on the various laboratory methods used to determine pathogenicity of staphylococci has not stressed the value of the plasma-coagulase reaction. Chapman<sup>1</sup> and his co-workers discussed the value of this test as compared to the hemolysis of blood, the pathogenicity for rabbits, and the agglutination test. These investigators, however, did not correlate the ability to coagulate plasma with the source of the strain. Their observations were made on five thousand strains of staphylococci with no classification as to their source. Fisher,<sup>2</sup> reporting on thirty strains isolated from human staphylococci lesions, found that all exhibited the ability to coagulate plasma.

The present study was made on 65 freshly isolated strains of staphylococci. Nineteen of these strains were isolated from patients with clinical manifestations

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†Cultures obtained from the Department of Bacteriology and Parasitology at the University of Chicago through the courtesy of Dr. G. M. Dack.

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of staphylococcus infection. Thirteen were collected from samples of raw milk, 24 were isolated from the air as contaminants, and nine were strains recovered from outbreaks of food poisoning in which the toxicity had been experimentally demonstrated.† All the strains of staphylococci used in this study fermented lactose and coagulated milk with acid production.

To determine chromogenicity, transplants were made on 1 per cent starch infusion agar so that well-isolated colonies were obtained. The plates were incubated at room temperature (27° to 30° C.) for three days before pigment formation was determined. Toward the end of this study, however, it was found that pigment production could best be observed by growing the staphylococci on milk agar plates. This medium was prepared by adding, aseptically, from a can of evaporated milk,\* 10 c.c. of milk to 90 c.c. of melted agar. The agar should be cooled to 50° C. before the milk is added. Pigment production was easily observed on this medium after the streaked plates had been incubated at 30° C. for three days.

To determine hemolytic activity, the staphylococci were cultured on extract agar plates in which 5 per cent defibrinated human blood had been incorporated. The observations were less variable on repeated tests with the use of human blood than with the use of rabbit blood.

The ability to coagulate plasma was demonstrated by inoculating 0.1 c.c. of a twenty-four-hour broth culture of staphylococci into 3 c.c. of diluted human plasma. The diluted plasma was prepared by adding one part of sterile physiologic salt solution to three parts of sterile citrated human plasma. The subcultures were incubated at 37° C. for eighteen hours before readings were recorded.

TABLE I

| STRAINS ISOLATED | SOURCE OF STRAINS | NUMBER PRODUCING PIGMENT | NUMBER HEMOLYZING BLOOD | NUMBER COAGULATING PLASMA |
|------------------|-------------------|--------------------------|-------------------------|---------------------------|
| 19               | Clinical lesions  | 16                       | 16                      | 19                        |
| 9                | Food poison       | 8                        | 8                       | 9                         |
| 13               | Raw milk          | 7                        | 7                       | 7                         |
| 24               | Air and dust      | 6                        | 1                       | 1                         |
| 65               | All sources       | 37                       | 32                      | 36                        |

Table I shows the results obtained from this study. Thirty-six strains were capable of coagulating plasma. All 19 strains isolated from patients with clinical manifestations of staphylococci infection coagulated the diluted plasma, while only one of the 21 strains isolated from the air and dust showed this characteristic. Another interesting observation was that the food poisoning strains of proved toxicity were all able to coagulate plasma. This reaction on further study with food poisoning staphylococci may prove to be valuable as an exclusion test in the epidemiologic study of such outbreaks. Only three of the 36 strains capable of coagulating plasma were the "albus" variety; one of these was isolated from milk of a cow with mastitis; another from a patient with an infected mammary gland, and the third was a food poisoning strain.

\*The evaporated milk used in these experiments was prepared by the Carnation Milk Company.

A number of samples of raw milk were cultured before thirteen strains of staphylococci were isolated. There is a chance that these were more or less saprophytic strains contaminating the milk during its production and distribution. However, two of them were isolated from the milk of a cow with definite symptoms of mastitis. Both of these strains coagulated plasma. It is possible that the other five coagulating strains of staphylococci recovered from raw milk might have been from cows with unrecognized symptoms of mastitis.

All 65 strains of staphylococci were retested for their ability to coagulate diluted human plasma after having been subcultured on extract agar for twelve months. The results obtained showed complete agreement in all cases with the readings made when the staphylococci were first isolated. Blair,<sup>3</sup> reporting on the stability of the plasma-coagulase reaction, recorded a 94 per cent agreement on the 102 strains tested between the original and the subsequent tests made two and one-half years later.

#### CONCLUSIONS

The ability of staphylococci to coagulate diluted human plasma is definitely correlated with its pathogenicity for man.

The ability to coagulate human plasma is apparently a stable characteristic of a strain of staphylococci.

The ability to coagulate plasma may prove to be valuable upon further study as an exclusion test in the epidemiologic study of staphylococci food poisoning outbreaks.

Milk agar prepared by the use of commercially manufactured evaporated milk is an excellent medium for determining chromogenicity of staphylococci.

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## THE MEASUREMENT OF FORMALDEHYDE FIXATION\*

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THE fixation of small protein structures by formaldehyde is effected by maceration in diluted formalin with or without suitable addition agents. In embalming, a similarly constituted solution is injected arterially. As the formaldehyde diffuses inward, it unites principally with the terminal  $\text{NH}_2$  groups of the protein structures, causing them to set to a more or less rigid gel. The degree of setting, hardness, or fixation of the preserved tissue is a matter of considerable importance, since it is usually desirable to maintain, as nearly as possible, the original consistency and appearance of the tissue. This is especially true in the case of dissecting material and in the embalming of cadavers. Formaldehyde is capable of greatly changing the consistency and gross appearance of tissues when used alone in aqueous solution, and it is, therefore, customary to add to it various other compounds designed to control or to direct its action. These concern mostly preservation and color. The various modifications of the Kaiserling process<sup>1</sup> constitute a case in point.

We have made quantitative studies of many of the physical and chemical factors involved in formaldehyde preservation under a grant from the Minnesota State Board of Health. The present report concerns the quantitative measurement of the hardness of formaldehyde-treated protein structures.

Since no commercial device was available for measuring hardness or rigidity of tissues on a numerical scale, we devised one. This instrument was a modification of one previously used by one of the authors<sup>2</sup> in other biological studies of physical and chemical factors in biology. In effect it measures the distance through which the surface of the specimen is depressed when a fixed force is applied to a small area of it.

### DESCRIPTION OF THE METER

Our meter was adapted from a 3-inch vacuum gauge and is shown in Fig. 1. The flexible ribbon and pipe connection were removed, and a tubular brass sleeve *S*,  $\frac{3}{4}$  inch by  $1\frac{1}{2}$  inches, carrying three short sturdy prongs *P* at its lower end, was fitted to the case. A  $\frac{1}{8}$ -inch brass rod *R* was then fitted to operate smoothly and without extra play within the sleeve. The upper end of the rod was attached to the lever system *L*, operating the pointer, and the lower rounded end of the rod was cut to such a length that it projected  $\frac{1}{8}$  inch beyond the plane of the sleeve prongs when the pointer was at zero. A normalized spring was then fitted to retain the rod in the extended position. The strength of the

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spring was such that a weight of 120 Gm. was required to displace the end of the rod into the plane of the three-sleeve prongs.

Similar devices may be constructed from small pressure gauges, opticians' diopter gauges, or dial reading auto tire gauges. It is essential to have a scale with large well-marked divisions, so that the readings can be taken at a single glance, and the stiffness of the spring must be suited to the range of pliability of the specimens to be measured. The spring which we made and used allowed a scale reading of only one division when the meter was applied to soft living tissues or fresh hog kidneys, and a reading of about 25 on embalmed dissection cadavers.

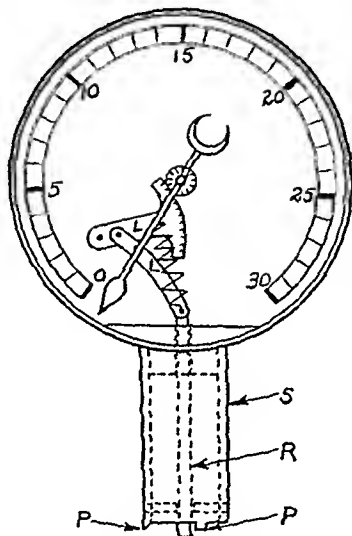


Fig. 1.—Fixation meter.

#### USE OF METER

In use, the protruding plunger of the meter is brought perpendicularly against the surface of the specimen and pressed carefully against it until the three-sleeve prongs barely touch the surface. The dial reading is then taken. Several readings over representative areas are taken and the results are averaged. Fleishy areas, which are more or less flat, are to be preferred since underlying bony structures affect the readings somewhat. By choosing like anatomic areas, consistent readings are easily obtained. In cadavers the abdomen and ventral surfaces of the thigh and forearm are preferred, since dependent areas are usually somewhat flattened, distorted, and otherwise changed.

#### APPLICATIONS

In living bodies the changes in muscle tonus during contraction and relaxation may be measured readily. The changes in tissue consistency accompanying the deposition of fat may be measured. The progress of rigor mortis with time is likewise measurable. The softening of structures following incipient putrefaction also may be followed. We have made hundreds of measurements with this meter upon various subjects, including man, in connection with our quantitative studies on the various phases of action of embalming fluid chemicals.

The time course of the so-called "embalming reaction," which occurs during and after the arterial injection of simple formaldehyde solutions, is shown graphically in Fig. 2. The curves show that the fixation begins when injection starts, increasing very rapidly, but not reaching a maximum until forty-eight hours or more have elapsed. So far as we are aware, no measurements like these have ever been reported, nor has any reference ever been made regarding a

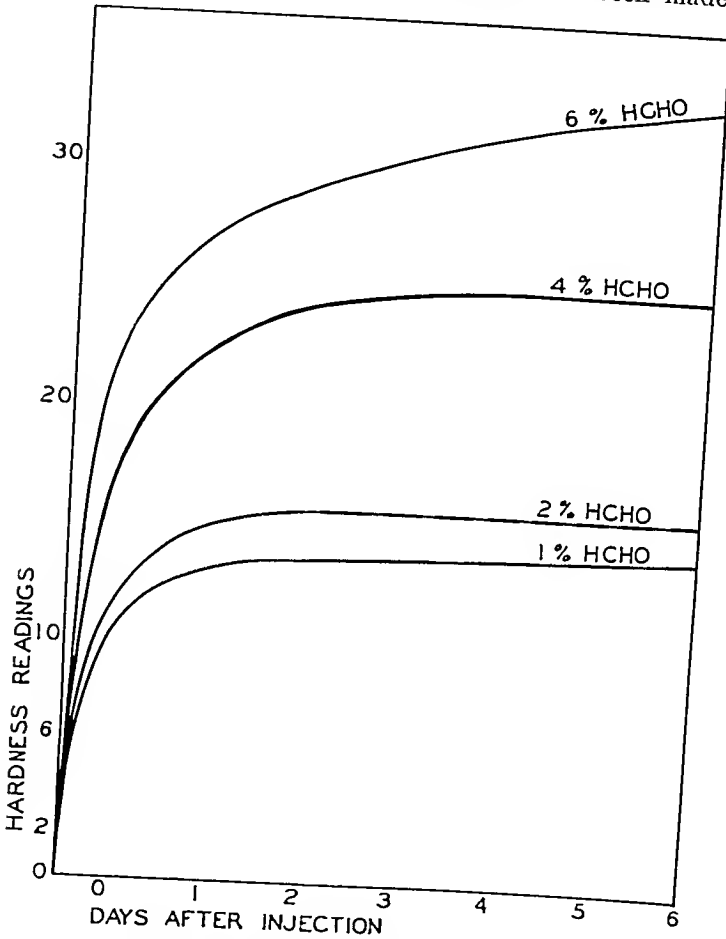


Fig. 2.—Time course of fixation reaction.

delayed fixing or embalming reaction. We believe that such postinjection hardness increases are due to a delayed action in which formaldehyde solutions of decreasing strength diffuse slowly into contact with still unattacked protein structures. Finally, the hardness becomes stabilized, but it may later decrease if the amount of formaldehyde used is so small that putrefaction begins.

The effect of increasing concentration of formaldehyde upon the hardness of tissue is also shown in Fig. 2. The curves represent the first published data upon the subject. All the curves show the time lag already mentioned. It is interesting to note that fixation does not increase directly with concentration. This suggests an adsorption phenomenon, but it may also be due to the "walling-

off" of the solution by local solid areas which interfere with the diffusion of the aldehyde. In time the compound may diffuse through, but then there is less of it. As a result the hardness is less than it would otherwise be. We are continuing the study of this point.

#### CONCLUSIONS

1. A simple portable meter has been devised for measuring the hardness of tissues, organs, or cadavers on a numerical scale.
2. Changes in the hardness of tissues under varying conditions and modes of treatment can be easily measured.
3. Only a few minutes of experience is needed in order to obtain consistent readings on hardness.
4. The use of the meter does not deface the anatomic structures in any way.
5. The instrument has proved useful in obtaining measurements regarding the synergistic effects of various compounds used in embalming fluids.
6. No numerical data of this kind are to be found in previous literature on the subject.

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#### A SIMPLE AND ACCURATE METHOD OF COUNTING SPERMATOZOA\*

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THERE are a number of various techniques in present-day general use that are employed in counting spermatozoa in a routine semen analysis. All these methods are complicated either by the use of blood counting pipettes or by the special diluting fluids. The method of Macomber and Saunders<sup>1</sup> involves the use of a blood-counting pipette (1:20 dilution) and a sodium bicarbonate-formalin solution (5 per cent sodium bicarbonate and 1 per cent formalin). The method of Hotchkiss<sup>2</sup> utilizes a diluent consisting of a solution of saturated sodium bicarbonate together with 1 per cent phenol. The "bulk" method of Belding<sup>3</sup> employs larger amounts of semen for dilution, but also makes use of the special Macomber-Saunders diluting fluid.

I use a much simpler method which obviates the necessity of pipettes, blood-counting pipettes, and special diluting fluids. This method is based on a recent report<sup>4</sup> which showed that plain, ordinary tap water is one of the most drastic

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spermicides known. The dilution with water serves not only to dilute the sperm cells so that they may be easily counted in a Neubauer blood-counting chamber, but it also causes immediate cessation of motion of the sperms facilitating the count. The use of tap water as the diluent is especially useful to those who have neither the time nor the facilities to prepare special solutions for an occasional semen analysis.

#### TECHNIQUE

One cubic centimeter of well-mixed whole semen is placed in an ordinary test tube, graduated at 20 c.c. Then ordinary cold tap water is added to the tube up to the 20 c.c. mark.

This simple technique has two distinct advantages: First, by simply adding water all the spermatozoa are killed at once; second, the use of a whole cubic centimeter of semen diluted 1:20 diminishes the error which crops up when using the small amounts of semen usually utilized in the blood-counting pipette technique.

The sperms are counted in the same fashion as are white blood cells, and the final count is arrived at in the following manner: After 1 sq. mm. is counted and rechecked (the sixteen small squares in one corner of the chamber), the number obtained is multiplied by 10 to constitute the number of sperms found in 1 c.mm. This is multiplied by 1,000 to find the number in 1 c.c. of diluted semen. To arrive at the number contained in 1 c.c. of whole undiluted semen, it is now necessary to multiply once more by 20 (the dilution used).

The above multiplication may be simplified by taking the number found in 1 sq. mm. and adding five zeros to it and multiplying by two.

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## THE USE OF THIOLYCOLLATE MEDIUM IN DIAGNOSTIC BACTERIOLOGY\*

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THE use of sodium thioglycollate in infusion medium was described by Brewer in 1940. This medium was described as a fluid medium in which anaerobes could be cultivated without the use of special fermentation tubes or other devices for excluding air. It was also pointed out that the thioglycollate inactivates most of the mercurials commonly used as preservatives in biologic products and is, therefore, useful in testing the sterility of such products. Marshall, Gunnison, and Luxen (1940) have confirmed its usefulness for such purposes. It has been found that this medium is well adapted for the growth of aerobic and micro-aerophilic types of bacteria as well. We have confirmed Brewer's reports and have made extensive use of this medium in routine bacteriologic examinations. After some time we observed that the results obtained by the use of this medium seemed to be better than those obtained with certain others in routine use. A survey of these results revealed the superiority of this medium for certain purposes, particularly in the culturing of specimens of blood and lochia.

This report includes examination of 69 consecutive blood specimens and 93 consecutive uterine specimens. Seven blood cultures were found positive by one or all of the methods used, and 82 uterine specimens were positive by one or more methods.

### TECHNIQUE

Blood specimens were, as a rule, planted at the bedside, but if this was not possible, the material was taken directly to the laboratory and placed into the various media within fifteen minutes of the time it was taken from the patient. Five to 10 c.c. of blood were plated directly into 50 c.c. of infusion broth containing dextrose. Duplicate plates were prepared for aerobic and anaerobic incubation, using 1.0 c.c. of the blood specimen in each plate. One cubic centimeter and 2.0 c.c., respectively, were added to tubes of thioglycollate medium. All cultures were incubated at 37° C. and inspected daily for ten days unless growth appeared earlier.

Uterine specimens were brought to the laboratory in Little's tubes wrapped in sterile towels. The contents were transferred to a tube of infusion broth, and inoculations of the various media were made by adding one or more drops of the material to each, depending upon the amount of lochia present. Blood agar plates for aerobic and anaerobic incubation, infusion broth, and thioglycollate medium were used. All media were incubated at 37° C. for ten days before being discarded as sterile, and all were examined daily. When growth appeared

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in broth or in thioglycollate medium, blood agar plates were made to obtain isolated colonies to be fished and studied in pure culture.

It should be noted that in many instances specimens were taken from patients being treated with one of the various sulfonamide compounds. While it has not been our experience (unpublished experiments) that the addition of such substances as p-aminobenzoic acid enhanced the ability of an infusion medium to produce positive blood cultures in such cases, the results that follow might indicate that if such compounds are useful for this purpose, they are to be found naturally in thioglycollate medium.

### RESULTS

Of the 69 blood specimens cultured, growth was obtained in only eight. No growth occurred in blood agar either aerobically or anaerobically in ten days. In four cases growth occurred in broth inoculated with 5 to 10 c.c. of the specimen, while growth was obtained in thioglycollate medium in seven cases. In only one instance did growth occur in broth when it did not appear in thioglycollate medium, yet in four cases growth was obtained in thioglycollate medium when it did not appear in broth, although the broth invariably contained from five to ten times as much inoculum. The types of organisms isolated were (1) from broth: alpha streptococci, three times; staphylococci, once; (2) from thioglycollate medium: alpha streptococci, four times; staphylococcus, twice; and pneumococcus, once. Growth appeared in these media as soon as twenty-four hours (in one case), or as late as the seventh day.

Of the 93 uterine specimens cultured, growth occurred on the blood agar plates incubated aerobically in 19 specimens, while those incubated anaerobically gave 16 positive cultures. Broth cultures were positive in 57 instances, and thioglycollate medium gave 82 positive cultures. An analysis of the results obtained with the various media is shown in Table I.

TABLE I

| Medium                 | POSITIVE CULTURES |    |    |   |    | TOTAL |
|------------------------|-------------------|----|----|---|----|-------|
|                        | 9                 | 39 | 10 | 7 | 17 |       |
| Thioglycollate medium  | 9                 | 39 | 10 | 0 | 0  | 82    |
| Broth                  | 9                 | 0  | 10 | 0 | 0  | 57    |
| Blood agar plates      | 9                 | 0  | 10 | 0 | 0  | 19    |
| (Aerobic incubation)   |                   |    |    |   |    |       |
| Blood agar plates      | 9                 | 0  | 0  | 7 | 0  | 16    |
| (Anaerobic incubation) |                   |    |    |   |    |       |

According to these data it would appear that thioglycollate medium has a superiority over broth in a ratio of 82:57; over aerobic blood agar plates the superiority is greater, 82:19. Compared with blood agar incubated anaerobically the ratio is 82:16. Since growth occurred in thioglycollate medium in all cases in which growth occurred in any medium when lochia was cultured, and only failed in one case when blood was being cultured—and in this case it is reasonable to assume that the larger inoculum used in the broth is accountable—it appears that thioglycollate medium has an important use in clinical and diagnostic bacteriology. No doubt some of the superiority lies in the fact that anaerobic as well as aerobic bacteria develop well in this medium.

While this paper was being written, *Neisseria intracellularis* (type III) was isolated from the spinal fluid of a case of meningococcus meningitis simultaneously on thioglycollate medium incubated aerobically and chocolate agar incubated in an atmosphere containing carbon dioxide. Isolation was not accomplished on unheated blood agar.

#### CONCLUSION

The use of thioglycollate medium by itself, or in conjunction with other media, is recommended in routine diagnostic bacteriology.

Since this paper was sent to the publisher this observation has been repeated in 5 additional cases of meningococcus meningitis. In 4 cases type I meningococcus was found, and in one, type II.

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The thioglycollate medium used in these studies was prepared by the Baltimore Biological Laboratory, Baltimore, Md.

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## MULTIPLE TISSUE WASHER AND PROCESSING ASSEMBLY\*

### COMBINATION APPARATUS FACILITATING THE PREPARATION OF TISSUES FOR PARAFFIN SECTIONS

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CAREFUL manipulation and preparation of relatively large numbers of tissue blocks as a laboratory routine are possible with a minimum of time, labor, and space with a combination multiple tissue washer and processing assemblies described here. This apparatus has been developed and improved as a matter of necessity for saving time, labor, and space in the course of preparing hundreds of tissues for paraffin sections. Careful consideration was given to histologic principles and methods as well as to the factor of minimum handling of the tissues with instruments.

The cost, availability of materials, and construction of the apparatus were given careful consideration. This apparatus can be constructed by a technician or student assistant with materials from the laboratory stockroom, hardware store, department store, woodshop, and the Kimball Glass Company, Vineland, N. J.

The combined apparatus, multiple tissue washer and processing assembly, is composed of the following parts:

1. Tissue washer: a deep, aluminum pan with 27 outlets and rubber tubes with Hoffman clamps (Fig. 1).

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\*From the Poultry and Chemistry Departments and Experiment Station, North Carolina State College of Agriculture and Engineering of the University of North Carolina, Raleigh. Aided by a grant from the North Carolina State College Research Fund.  
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2. Processing assemblies: inverted, open-bottom, screw-cap vials, perforated caps and corks fitting into fruit juice glasses (Fig. 2).
3. Tripod: japanned iron tripod (Fig. 1).
4. Assembly trays: eypress or aluminum (Fig. 1).

The size of the tissue washer, the number of processing assemblies, and the size of the assembly trays will depend chiefly on the requirements of the laboratory. A sufficient number of assemblies should be provided to permit a serial progression of several units of assemblies with tissues through the steps of fixing, washing, dehydrating, clearing, and paraffin infiltrating. In this laboratory, using a tissue washer with 27 tubes, 46 assemblies are used for the first four steps in the procedure and 30 additional assemblies are kept in the paraffin oven for infiltrating the tissues.

*The Tissue Washer* (Fig. 1).—The multiple tissue washer consists of a deep, 20-gauge, aluminum pudding pan of 6 quarts capacity, perforated with 27 holes jointed to short copper tubes holding 7-inch length rubber tubes provided with Hoffman clamps. The size of the pan and the number of outlets will depend on the requirements of the laboratory. The 27-outlet washer in use in this laboratory has two circular, inner and outer, rows of holes drilled through the bottom of the pan. The outer row contains 18 holes equally spaced. The inner row has 9 holes equally spaced, and each hole is placed opposite one in the outer row. The holes in the inner row should be placed opposite the spaces between the holes in the outer row. The holes are drilled from the outside to the inside. Soft copper tubing,  $\frac{1}{4}$  inch outside diameter is cut into one-inch lengths and forced into the holes. These holes are made slightly smaller to hold the tubes firmly in place until jointed with liquid solder. The height of the tubes inside the pan is about  $\frac{3}{16}$  inch above the bottom to provide anchorage for the solder. Care is taken that the tubes are perpendicular to the bottom of the pan. Liquid solder is spread around each tube level with its upper end. It is allowed to harden overnight. To further strengthen the support of the tubes in the outer row, which is in a peripheral moat, a ribbon of solder is laid in the moat. Each tube is surrounded by this ribbon of solder. The inner edge of the ribbon reaches the inner rows of tubes, strengthening them likewise. The solder is allowed to harden overnight. The pan is inverted on the tripod, and liquid solder is spread around the copper tubes, strengthening them still more. This tissue washer has been in constant use for more than a year, and not a single tube has loosened or been resoldered. The ribbon of solder in the moat has contracted and drawn away from the wall of the pan, but it has not affected the use of the washer in any way. A rubber tube, 7 inches in length,  $\frac{1}{16}$  inch wall,  $\frac{1}{4}$  inch bore, is placed over each copper tube, and a Hoffman clamp,  $\frac{1}{2}$  inch size, is clamped onto each rubber tube.

This multiple tissue washer possesses the following advantages:

1. It is durable, inexpensive, and easily constructed.
2. It provides a reservoir of water from a single tap for one or 27 assemblies.



3. It provides a constant flow of water, the rate of which is controlled by the Hoffman clamp, and can be regulated to a full stream or to a definite number of drops per minute.

4. It requires only a very small amount of space for operation and storage.

*The Processing Assembly* (Fig. 2).—The processing assembly consists of an inverted, open-bottom vial, a molded bakelite cap perforated with 5 to 7 holes, a plain 5-ounce fruit juice glass, and two corks,  $2\frac{1}{2}$  by  $\frac{5}{8}$ , and 1 by  $\frac{1}{2}$  inches in size. The larger cork fits into the glass container, and has a center hole of a size to hold the vial firmly and upright in place, yet permitting easy shifting of its position in the assembly. The smaller cork is used as a stopper in the

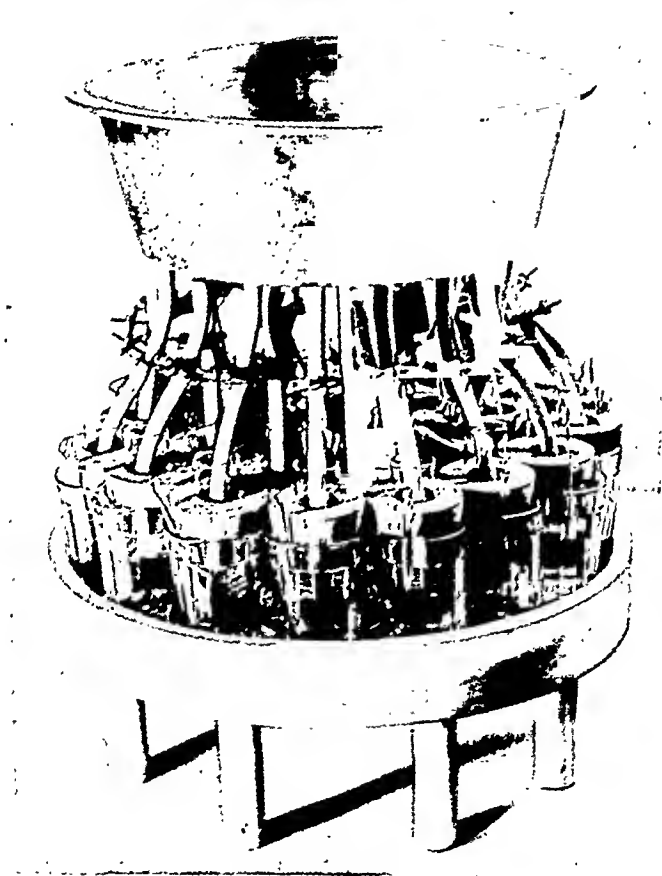


Fig. 1.—Photograph of multiple tissue washer and processing assemblies with assembly tray.

top opening of the vial. The vial, size No. 4,  $4\frac{1}{4}$  inches in length, is cut down to a length of about 4 inches. This is accomplished by winding a double loop of a stout cord around the bottom of the vial, and with a see-saw movement of the loose ends of the cord create sufficient friction and heat to cause it to break clean when plunged into ice water. The sharp edges are made smooth by glazing over a Bunsen burner. The molded Bakelite cap is perforated with 5 to 7 holes, the size and number of which will depend on the kind and size of tissues routinely prepared. There should be several perforations in the

cap to permit a free movement of the convection currents into and out of the lumen of the vial, thus assuring a more perfect interchange of reagents between the tissue and the surrounding medium.

*The Tripod* (Fig. 1).—The iron tripod is of heavy material. The outside diameter of the ring is  $5\frac{1}{8}$  inches. The height of the tripod which supports the washer is 9 inches. The diameter of the circle formed by the inner row of tubes on the bottom of the washer should be approximately 7 inches to allow the ring top of the tripod to fit within this circle.

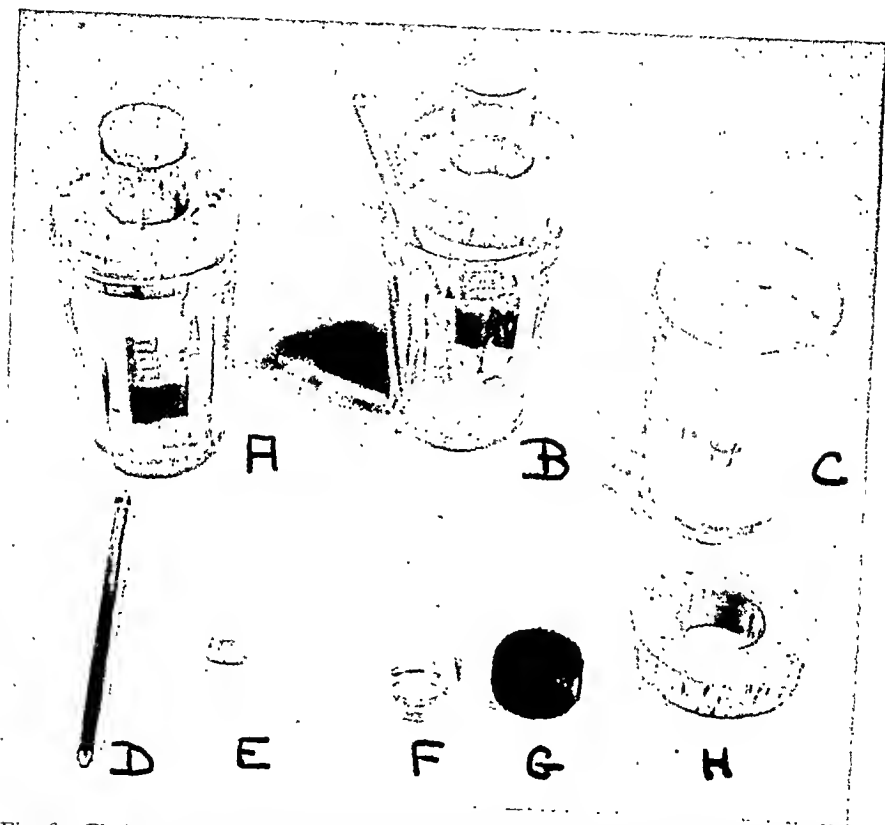


Fig. 2.—Photograph of a processing assembly and its parts. (A) Assembly complete for fixing and dehydrating tissues. (B) Assembly complete for washing tissues. (C) Assembly glass. (D) Glass rod. (E) Small cork stopper for vial. (F) Open-bottom vial. (G) Perforated bakelite cap, for vial. (H) Large cork with center hole for holding vial.

*The Assembly Trays* (Fig. 1).—The number of assembly trays is dependent on the size of the washer and the number of assemblies used. If only a few are used, a small tray will be satisfactory, but if 27 or more assemblies are used at one time a large tray will be required. Even though the larger number of assemblies is maintained, the smaller tray will be found very convenient when only a few assemblies are in operation. The small tray is a 22-gauge, aluminum layer-cake pan, 10 inches in diameter with a  $\frac{3}{4}$  inch hole in the wall to serve as an outlet.

A large tray to hold 27 assemblies was designed and constructed from a single piece of eypress, 2 inches in thickness. It was turned on a lathe to form

a tray with an inside diameter of 14 inches, a rim 1 inch high and  $\frac{1}{2}$  inch wide, making a total diameter of 15 inches. There are eight 4-inch legs,  $\frac{3}{4}$  inch in thickness, supporting the tray. The tray was designed to be used in a sink.

A second large tray was designed to be used on top of the counter. A wide, shallow "Wear-Ever" aluminum cream pan, No. 401, bottom diameter  $14\frac{3}{4}$  inches, top diameter  $16\frac{5}{8}$  inches, depth  $3\frac{1}{4}$  inches, was purchased from a hardware store. The pan has a single, large outlet, with a rubber tube attached to carry off the overflow water from the assemblies. This outlet was made by drilling through the wall of the pan a hole  $\frac{3}{4}$  inch in diameter,  $\frac{1}{4}$  inch from the bottom. A piece of copper tubing,  $\frac{3}{4}$  inch in diameter,  $1\frac{1}{2}$  inches long, was used for the overflow pipe. At one end of the tube a flange was made by making several longitudinal cuts to a depth of  $\frac{1}{4}$  inch, and pressing the segments outward. The tube was forced through the hole, and the flange was shaped to the curvature of the wall of the pan. The tube was soldered to the wall, inside and outside, of the pan. After hardening, a rubber tube  $\frac{3}{4}$  inch, inside diameter, 1 foot in length, was slipped over the copper overflow pipe.

The durability and practicability of these two large assembly trays have been thoroughly tested and found satisfactory.

#### ADVANTAGES OF THE PROCESSING ASSEMBLY

The processing assembly is used to carry the tissues through fixation to paraffin imbedding with a minimum handling of the tissues. The principal feature of the assembly is the vial and the perforated bakelite cap, fitting tightly in the center of the hole of the large cork. When this unit is in place, the vial is centered and its distance from the bottom can be changed by shifting its position in the cork. The centering and manipulation of the vial have many advantages.

1. It serves to suspend the tissue in the fixing fluid and permits the penetration of the fixative through all surfaces.
2. It permits thorough washing of the tissue. A rubber tube of the washer is placed inside the vial through the top. A glass rod is placed in the container between the cork and the top edge of the glass.
3. The flow of water through the tube from the reservoir of water is controlled and regulated by the Hoffman clamp.
4. A full stream or only a few drops of water per minute, depending on the character of the tissue, is permitted to flow into the vial.
5. The water circulates in the vial and flows out into the container and over its edge.
6. The glass rod keeps the cork away from one side of the glass, permitting this overflow of water.
7. A continuous flow of fresh water is possible.
8. The loss of the tissue from the vial is prevented as the level of the water inside the vial is below the top opening of the vial.
9. The processing assembly permits the use of calcium oxide and dioxane as dehydrating agents.
10. The vial can be raised above the lumps of calcium oxide.

11. The perforated cap permits the interchange of dioxane and water between the tissue and fluid and the passage of water down to the calcinm oxide.
12. The assembly permits dehydration of the tissue with a series of graded alcohols.
13. It permits infiltration of the tissues with paraffin.
14. It permits easy handling of the tissues in the process of imbedding.

A second assembly, kept permanently in the paraffin oven, is used with distinct advantage in this step. The melted paraffin may be kept in the assembly or placed in it prior to transferring the tissue from the first unit. Then the tissue is transferred without handling by inverting the first vial and dropping the tissue into the paraffin in the second vial. The suspension of the tissue in the paraffin allows the paraffin to penetrate into the tissue through all its surfaces, the assembly permits easy handling of the tissue in the process of imbedding. The unit with the paraffin-infiltrated tissue is taken to the imbedding box, and there the tissue is transferred to liquid paraffin in a glass stain jar. The transfer is accomplished by grasping the cork, holding the vial and inverting it, thus avoiding getting hot paraffin on the hands. Occasionally it may be necessary to unscrew the cap to remove the tissue. Placing the tissue in the glass stain jar permits the technician to ascertain its size and shape, thus determining its orientation in the imbedding block. Then the tissue may be transferred to the imbedding block with a perforated spoon.

#### LIST OF MATERIALS

Capsule vials, screw cap, size No. 4, No. 60957.

|                              |                     |         |
|------------------------------|---------------------|---------|
| Height $4\frac{1}{4}$ inches | $\frac{1}{2}$ gross | \$ 3.75 |
| Black molded caps            | $\frac{1}{2}$ gross | 1.00    |

Purchased from Kimball Glass Company, Vineland, N. J.

The prices listed are given only to show cost at time of purchase. The following materials may be purchased from any laboratory supply house, hardware store, department store, and woodshop.

|  |        |
|--|--------|
| Hoffman clamps, each   | \$ .20 |
| Tripod   | .55    |
| Rubber tubing, per foot  | .08    |
| Copper tubing, $2\frac{1}{2}$ feet   | .30    |
| Aluminum pudding pan, 20-gauge, 6-quart capacity                                   | 1.25   |
| Aluminum layer-cake pan, 22-gauge, 10-inch diameter                                | .60    |
| Liquid metallic solder, tube   | .10    |
| Corks, XXX quality, $2\frac{1}{4}$ by $2\frac{1}{8}$ by $\frac{3}{8}$ , bag of 100 | 10.55  |
| Corks, XXX quality, 1 by $\frac{7}{8}$ by $\frac{1}{2}$ , bag of 100               | 2.35   |
| Assembly tray, cypress   | 4.50   |
| Assembly tray, aluminum cream pan, No. 401   | 3.00   |
| Glass rod, No. 6, 10.5 ft.   | .25    |

Approximate cost of tissue washer with 27 outlets:

|                          |         |
|--------------------------|---------|
| Aluminum pan             | \$ 1.25 |
| Copper tubing and solder | .75     |
| Rubber tubing, 16 feet   | 1.28    |
| Hoffman clamps, 27       | 5.40    |
|                          | \$ 8.68 |

Approximate cost of 72 processing assemblies:

|                                    |             |
|------------------------------------|-------------|
| Fruit juice glasses, 6 dozen @ .40 | \$ 2.40     |
| Screw-cap capsule vials, 6 dozen   | 3.75        |
| Black molded caps, 6 dozen         | 1.00        |
| Corks, large, 6 dozen              | 7.56        |
| Corks, small, 6 dozen              | 1.69        |
| Glass rods, 27                     | .25 \$16.65 |
| Each assembly: \$0.231             |             |
| Tripod                             | .55         |
| Small assembly tray, aluminum      | .60         |
| Large assembly tray, cyprus        | 4.50        |
| Large assembly tray, aluminum      | 3.00        |

Approximate cost of combined apparatus, \$29.48 to \$30.98.

## CHEMICAL

### BLOOD BROMIDE DETERMINATIONS: THEIR USE AND INTERPRETATION\*

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THE critical level at which symptoms of bromide intoxication appear is the subject of considerable controversy. In addition to its diagnostic value the blood bromide determination has been used in estimating prognosis. This has led to conflicting statements, since many writers have attempted to correlate the symptoms of bromide intoxications with an absolute figure in terms of bromide content of blood and cerebrospinal fluid. Such disagreement is due to the use of chemical methods which do not selectively and accurately determine bromine, and to personal differences among patients which condition the absorption and excretion of bromides. It is the purpose of this paper to present data obtained by the most popular methods, to discuss sources of error and the biochemical and physiologic factors which are pertinent to the interpretation of the blood bromide concentration when used in diagnosis and treatment.

#### METHODS FOR DETERMINING BROMIDES IN BODY FLUIDS

Bromine is the most difficult halogen to determine, particularly in the presence of others. The exact chemical methods, whether volumetric or gravimetric, require elaborate equipment and laboratory facilities beyond those possessed by most hospitals and skill beyond that of the average laboratory technician. For the most part they require from six to twenty-four hours for completion; the disadvantage of this element is obvious when the results are needed in emergency situations.

Most clinical investigations of blood bromides have been made by adding gold chloride to a protein-free filtrate of serum. If bromide is found beyond that normally present (0.7 to 1.0 gamma per 100 c.c.), there is a color change in the mixture from yellow to brown (or even to brownish red) due to the formation of gold bromide, and the depth of color is proportionate to the concentration. This reaction is inversely analogous to that which occurs in vivo after the ingestion of bromides when the bromide ion is substituted in the body for the chloride ion. The gold chloride reaction was first used by Walter.<sup>1</sup> Walter's method was said to be specific for bromides, but was later shown to be affected by other halogens. Wuth<sup>2</sup> and Diethelm<sup>3</sup> further modified the procedure, recommending it for the diagnosis of bromide intoxication. Diethelm used color tubes containing sodium bromide treated with gold chloride and trichloroacetic acid as standards and with them the comparison was made without optical assistance.

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Fremont-Smith, Dailey, and Sloan<sup>4</sup> compared Walter's method with that of Toxopeus<sup>5</sup> and found that there were large discrepancies in their results.

Wuth stated in his paper that there was considerable inherent error due to the adsorption of bromide by the serum proteins. This, he claimed, was of the order of 1 to 2 per cent in concentration of 25 mg. per 100 c.c., and from 11 to 14 per cent in a concentration of 200 mg. per 100 c.c. Katzenelbogen and Goldsmith,<sup>6</sup> who used modifications of the Walter test, experienced difficulty in matching the standard gold bromide with that in the serum. They attempted to obviate this by adding sodium chloride to the standard solution.

Without question the gold chloride method determines bromides, but, in addition, it may determine other substance as well, and thereby lose one of the chief values of a chemical examination, i.e., specificity.

Yates<sup>7</sup> procedure is probably the simplest accurate method which does not require equipment beyond the scope of a good clinical laboratory. A protein-free preparation of the material being analyzed is placed in a nickel crucible with 25 per cent potassium hydroxide and evaporated to dryness. It is heated in an oven at 500° C. for twenty minutes, then cooled to 18° C. Sulfuric acid is added slowly, followed by a chromic-sulfuric acid mixture. Potassium iodide and starch solution are added, and the mixture is then aerated for five hours. At the end of that time, it is titrated with N/1,000 sodium thiosulfate. We have obtained consistent results and excellent recoveries with this method.

#### EXPERIMENTAL PROCEDURE

In the course of routine bromide determinations we have observed that the gold bromide reaction is subject to considerable error and, in spite of its simplicity, the potential error is enormous. Our experiments were devised to ascertain whether the gold chloride method would (1) yield duplicate results on the same material, (2) negative results in the absence of bromine, and (3) recover added bromides from serum. The effect of varying serum protein concentrations and of varying the bromide concentrations with respect to protein adsorption was also studied.

The procedure for blood bromide determinations followed in our experiments was that suggested by Diethelm, but three quantitations were made on each specimen of blood, employing for standard comparison (1) color tubes containing various dilutions of sodium bromide treated with gold chloride and graduated at intervals of 10 mg.; (2) a Bausch & Lomb visual type colorimeter, using standard solutions of gold bromide containing 1 or 2 mg. of sodium bromide per cubic centimeter according to the expected concentration of bromide in the unknown solution, and (3) a photoelectric comparator which had been standardized with ten solutions of sodium bromide treated with gold chloride. Using this apparatus, it was possible to refer all bromide concentrations to a single standard. The standard solutions were checked by the method of Yates for accuracy. The mean error in them was  $\pm 0.78$  per cent. The bloods were filtered after being treated with trichloroacetic acid to remove proteins. There were added to the filtrates, 0.5 per cent gold chloride in the proportion of 0.2 c.c. to each 1 c.c. of filtrate.

A series of bloods known to contain increased amounts of bromides were examined by the foregoing procedures. The findings were compared to determine whether it is possible to obtain duplicate values on the same specimen and to learn the error between several methods of comparing unknown concentrations of gold bromide with standard concentrations. The blood specimens were obtained from voluntary subjects of other experiments, who received sodium bromide by mouth and no other medication for periods ranging from ten days to six weeks. The ordinary diet with customary sodium chloride was eaten. Two determinations by each method were done, and each specimen was quantitated by the three methods described. The data from 20 specimens are presented in Table I, from which it can be seen that the visual comparison of gold chloride treated blood filtrates with color tubes is accompanied by considerable error. This is chiefly due to the impossibility of matching colors closely by this means, and in part to the fact that our color tubes represented intervals of 10 mg. of bromide. Using these tubes, closer intervals than 5 mg. could not be approximated by the eye. The range of values found by visual comparison under standardized illumination was from 20 to 155 mg. per 100 c.c., and the average difference between two determinations on the same serum was 19.8 per cent. The maximum difference was 37.5 per cent and the minimum was in one case 0.

When a Bausch & Lomb colorimeter was used for comparison, the bromide values could be estimated more closely. The range of concentration was from 21.4 to 156.4 mg. per 100 c.c. A number of specimens could not be matched exactly with the standard, appearing to be slightly off-color (Katzenelbogen and Goldsmith<sup>6</sup>). The average difference between two determinations was 6.1 per cent, the maximum 19.8 per cent and the minimum 0.4 per cent. Thus the use of the colorimeter reduced the error considerably, but the analyst is frequently confronted with bloods in which a comparison is very difficult or impossible even with this optical assistance. Except for the badly matching filtrates, the difference was 5.5 per cent or less, which limit is a considerable error for exact chemical determinations, but is much more acceptable than the values obtained by direct visual comparison.

The use of the photoelectric colorimeter further reduces the variation between two determinations on the same blood. The average difference in the twenty bloods reported here was 0.7 per cent. The maximum difference was 2.3 per cent and the minimum was 0. For clinical purposes this instrument offers relatively little advantage over the Bausch & Lomb colorimeter, but it entirely eliminates the individual factor in making comparisons; it allows reference of all specimens to a single standard and permits many determinations to be made in a very short time.

However, the close agreement between some of these determinations when made by colorimeter may be misleading, since in many laboratories the only criterion for control of a method is that it shall yield comparable results on the same material. The possibility of chlorides and other substances being determined along with bromides is not ruled out when such a criterion is used.



With respect to the use of prepared color tubes for comparison, as in Diethelm's procedure, it is probable that the error would be greater than that observed by us, since the intervals recommended are greater than 10 mg. at many points.

#### INFLUENCE OF SUBSTANCES OTHER THAN BROMIDES

A number of bloods were taken from patients who had been hospitalized for a long time and who had not received any bromide medication. These were subjected to the specific qualitative test of Belote,<sup>8</sup> and a gold chloride determination was made. As far as possible bromides were ruled out of the immediate past history of these individuals, either from the hospital record or from their own statement. Among the 76 bloods examined were several from reliable voluntary subjects who disclaimed bromide ingestion for six months or longer before the blood was drawn. Of this series no blood gave a positive qualitative eosin reaction, but 9 showed from 16 to 53 mg. of bromide by the gold chloride method. Two patients took no medication of any sort for four and six months, respectively, and the other 7 received various substances. Among the medication received by the patients who appeared to have increased blood bromides were the following substances: cysteine, diphenylhydantoin, liver extract, caffeine sodiobenzoate, wine of Bulgarian belladonna, thyroid, pituitary extract, and physiologic saline solution.

It is difficult to explain the positive findings in these cases unless the gold chloride reaction is nonspecific. It has been pointed out by Johnson and Partlow,<sup>9</sup> Tod,<sup>10</sup> and Katzenelbogen and Czarski<sup>11</sup> that chloride and iodides interfere with the reaction; this may explain the findings in several patients. If it is true that the gold chloride reaction is nonspecific, levels at which toxic symptoms appear must be disregarded, since some of the so-called blood "bromides" in many cases may actually be due to nonbromide material. Thus the use of the gold chloride reaction to follow the bromide treatment of epileptic patients with bromides may be fallacious.

To corroborate the positive findings in patients with blood bromides who had not been ingesting bromides, the medication which they had been receiving was added to bromine-free blood sera (1:20,000) and gold chloride tests were made. Diphenylhydantoin, pituitary extract, thyroid, potassium iodide, and Lugol's solution gave negative results by the eosin test, but produced slight degrees of color after gold chloride was added. The color produced was not adequate for comparison with a standard by a colorimeter, but the observation suggests that the gold chloride reaction is nonspecific for bromides.

#### ADSORPTION OF BROMIDES BY BLOOD PROTEINS

If we assume that the error which tends to lower the bromide values is chiefly due to protein adsorption, it would seem probable that the higher the concentration of proteins in the serum, the more adsorption would occur. Twenty bromine-free sera to which sodium bromide in concentrations varying from 25 mg. per 100 c.c. to 600 mg. per 100 c.c. had been added were examined. The total serum proteins were determined by the method of Greenberg;<sup>12</sup> the bromide concentration was determined by the Yates method and by the gold chloride method.

TABLE I

REPRODUCIBILITY OF RESULTS BY DIFFERENT METHODS OF COMPARISON WITH STANDARD

| SPECIMEN NO.     | DIRECT COMPARISON WITH COLOR TUBES |                     | BY USE OF BAUSCH & LOMB COLORIMETER |                     | BY USE OF PHOTOELECTRIC COLORIMETER |                     |
|------------------|------------------------------------|---------------------|-------------------------------------|---------------------|-------------------------------------|---------------------|
|                  | MG. PER 100 C.C.                   | PER CENT DIFFERENCE | MG. PER 100 C.C.                    | PER CENT DIFFERENCE | MG. PER 100 C.C.                    | PER CENT DIFFERENCE |
| A 3              | 70                                 |                     | 74.3                                |                     | 74.3                                |                     |
|                  | 95                                 | 26.3                | 76.2                                | 2.4                 | 74.7                                | 0.5                 |
| A 7*             | 125                                |                     | 127.0                               |                     | 127.4                               |                     |
|                  | 150                                | 20.0                | 146.2                               | 13.1                | 127.0                               | 0.3                 |
| A 8              | 65                                 |                     | 72.7                                |                     | 73.4                                |                     |
|                  | 80                                 | 23.3                | 73.8                                | 1.5                 | 73.9                                | 0.6                 |
| A 9              | 50                                 |                     | 58.1                                |                     | 58.0                                |                     |
|                  | 65                                 | 30.0                | 58.4                                | 0.5                 | 58.0                                | 0                   |
| A 10*            | 70                                 |                     | 68.6                                |                     | 68.9                                |                     |
|                  | 85                                 | 17.6                | 59.8                                | 12.6                | 68.6                                | 0.4                 |
| A 11*            | 120                                |                     | 156.4                               |                     | 141.9                               |                     |
|                  | 155                                | 22.5                | 140.0                               | 10.4                | 145.3                               | 2.3                 |
| A 14             | 70                                 |                     | 88.8                                |                     | 90.1                                |                     |
|                  | 90                                 | 22.2                | 92.4                                | 3.3                 | 90.1                                | 0                   |
| A 16             | 70                                 |                     | 63.4                                |                     | 59.9                                |                     |
|                  | 55                                 | 21.4                | 59.9                                | 5.5                 | 60.1                                | 0.3                 |
| A 21             | 75                                 |                     | 87.8                                |                     | 83.2                                |                     |
|                  | 90                                 | 16.6                | 84.3                                | 3.9                 | 83.7                                | 0.5                 |
| A 29*            | 90                                 |                     | 125.3                               |                     | 106.9                               |                     |
|                  | 110                                | 18.1                | 107.0                               | 14.6                | 106.9                               | 0                   |
| A 30             | 115                                |                     | 136.3                               |                     | 136.3                               |                     |
|                  | 135                                | 14.4                | 130.0                               | 4.6                 | 139.2                               | 2.0                 |
| A 31             | 115                                |                     | 119.0                               |                     | 117.4                               |                     |
|                  | 140                                | 17.8                | 116.3                               | 1.3                 | 118.0                               | 0.5                 |
| B 13             | 55                                 |                     | 55.4                                |                     | 55.2                                |                     |
|                  | 65                                 | 15.4                | 57.8                                | 4.1                 | 55.4                                | 0.4                 |
| B 14             | 70                                 |                     | 67.2                                |                     | 66.2                                |                     |
|                  | 70                                 | 0                   | 66.1                                | 1.6                 | 66.6                                | 0.6                 |
| B 15             | 80                                 |                     | 98.4                                |                     | 95.9                                |                     |
|                  | 110                                | 37.5                | 98.6                                | 5.3                 | 96.3                                | 0.4                 |
| B 16             | 20                                 |                     | 21.4                                |                     | 22.4                                |                     |
|                  | 30                                 | 33.3                | 26.7                                | 19.8                | 22.9                                | 2.0                 |
| B 41             | 45                                 |                     | 47.4                                |                     | 47.6                                |                     |
|                  | 55                                 | 18.1                | 48.3                                | 1.0                 | 48.1                                | 1.0                 |
| B 43             | 80                                 |                     | 87.6                                |                     | 89.3                                |                     |
|                  | 95                                 | 15.7                | 88.0                                | 0.4                 | 90.1                                | 0.8                 |
| B 44             | 100                                |                     | 103.5                               |                     | 102.4                               |                     |
|                  | 105                                | 4.7                 | 105.7                               | 1.1                 | 103.1                               | 0.6                 |
| B 45*            | 80                                 |                     | 129.6                               |                     | 111.6                               |                     |
|                  | 115                                | 21.7                | 112.4                               | 14.2                | 110.6                               | 0.8                 |
| Average per cent |                                    | 19.8                |                                     | 6.1                 |                                     | 0.7                 |

\*These bloods could not be matched easily with standard.

It was found that the protein concentration has little relation to the amount of bromide recovered, since the values were almost evenly distributed with respect to the mean protein value and the mean bromide recovery value.

When recovery values were plotted against bromide concentration, it appeared that the higher the concentration the lower was the recovery of bromides from serum by the gold chloride method. Thus, it could seem that the error is greatest when exact information is most important due to the critical implications of high bromide values. No marked effect on the gold chloride method was observed by altering the acidity of either unknown or standard solutions within the limits which might be expected in the course of an ordinary determination.

#### DISCUSSION

The use of methods which give only an inaccurate estimate of the bromine concentration is unfortunate. The gold chloride method is not accurate, and its use for the initial diagnosis of intoxications may be adequate, but a simple, accurate method is needed for use in prognosis and in following treatment. It must be employed before an attempt to correlate the symptomatology with blood concentrations in bromide intoxication can be made.

This oversight may lead to more serious confusion than already exists. As a medicolegal test the value of the gold chloride method would be seriously questioned but the failure to consider individual variations in the ability to handle bromides is possibly more serious than the inaccuracies of the method. The danger of confusing bromide intoxications with other conditions has been pointed out frequently, but no one has expressed concern over confusing other stuporous conditions with bromide intoxication and administering large amounts of salt. We should consider the folly of giving 20 Gm. of salt daily to a person who has suffered head trauma and who has taken bromides to relieve the pain of a concussion or a subdural hemorrhage, or to one who is suffering from coma of renal origin. Elevated bromide contents in the blood are often found superimposed upon more serious conditions resulting from entirely different etiology.

In dealing with conditions in which bromides are represented, the determination of urine and blood chlorides, along with bromides, is a far better indication of the progress of treatment or of the validity of a diagnosis of bromide intoxication than isolated blood bromide determinations, but the clinical condition of the patient is more important than any laboratory finding. It is not our belief that blood bromide determinations should be abandoned, but rather that more accurate methods should be employed and that the limitations of laboratory findings should be recognized.

#### SUMMARY

Blood bromide concentrations are usually determined by a reaction in which gold chloride is added to a protein-free filtrate of serum, and the resulting colored substance is compared with quantitative standards. Most of the exact bromide methods are difficult and are beyond the scope of hospital and clinical laboratories. The gold chloride method has been examined critically, and sources of error are described. It is possible to obtain almost duplicate results

with some modifications of this method, but this is misleading, since it causes unwarranted faith to be placed in the results regardless of other sources of error. The use of a colorimeter can reduce the error considerably, but it has been our experience that it is appreciable. The reaction is probably not specific for bromides, since other chemically unrelated substances may cause a positive reaction. The concentration of blood bromides appears to be more important than the serum-protein concentration as a source of error. There have been reported anomalous findings of persons with extremely high blood bromides who showed no toxic symptoms, and of others who have demonstrated extreme bromide intoxication while the blood bromide was low, which may be related to the method used. The comparison of chloride and bromide levels in blood and urine is more valuable than isolated determinations of the blood bromide. Such data should be used as presumptive evidence. The clinical condition of the patient is a more valuable diagnostic and therapeutic index.

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## STUDIES ON MELANURIA\*

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MELANURIA usually occurs in the final metastasizing stage of malignant melanomas. In the urine a colorless or light-colored precursor of melanin, a water-soluble melanogen or chromogen, is excreted which in the presence of oxygen is readily transformed into the dark melanin precipitate. The usual laboratory tests to detect melanuria are based on this fact. An oxidizing agent is added to the urine, and the subsequent darkening is taken as a sign of melanuria. The recommended oxidizing agent is ferric chloride. As a second test bromine water is used. Most biochemical and clinical laboratory manuals and textbooks do not mention tests other than those for detection of melanin precursors. These tests, however, are neither sensitive nor specific, and they yield highly confusing and inconsistent results, as pointed out by Blackberg and Wanger in 1933.<sup>1</sup> Ferric chloride may form dark brown precipitates in normal urines if they are alkaline, and falsely positive results also can be obtained if drugs are excreted in the urine. Furthermore, these tests are hardly more sensitive reactions than the simple darkening of the urine exposed to air.

And yet, more sensitive and more specific qualitative chemical tests for the detection of melanogen have been known since 1887. In that year Thormählen<sup>2</sup> described the reaction which has been named after him, and which in our laboratory was found to be about 50 times as sensitive as the oxidation tests. So far the Thormählen test has never been found to be positive in the urine in any condition other than melanuria.

This test is performed in a manner similar to the Legal test for acetone, although it has no relation to it. A few drops of diluted solution of sodium nitroprusside are added to 3 to 5 c.c. of the urine, and then a few drops of a strong alkali solution. In our experience 10 per cent sodium hydroxide solution is the best alkalizing agent, better than ammonia or caustic soda. By alkalization a deep ruby color results. This color is not characteristic for the melanogen. It is also formed by acetone and by creatinine. But if one acidifies with glacial acetic acid an azure blue color immediately appears which is specific for the melanogen. By addition of glacial acetic acid acetone turns deeper red; creatinine becomes yellow, then slowly green, and finally blue. Of course, Thormählen's test can be considered positive only if, through acidification, the color suddenly changes to blue.

Another even more sensitive, but less specific, test is Ehrlich's aldehyde reaction, which is known as the urobilinogen test. There is evidence that the

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positive Ehrlich reaction in melanuria is not due to the simultaneous presence of urobilinogen. The urobilinogen reaction disappears from the urine after its exposure to air for a short time (maximum after two hours), whereas in melanuria the test remains positive as long as does the Thormählen test, usually for several days. In fractionating the urine by different precipitation methods, the Ehrlich test is positive in those fractions which contain the melanogen, and negative in the others. Even preparations of melanogen which are purified chemically to a high degree are Ehrlich positive.<sup>3</sup>

The Ehrlich test was found in our laboratory to be about twice as sensitive as the Thormählen test. Therefore, melanogen may be detected with Ehrlich's reagent when the Thormählen reaction is still negative. However, in such a case the Ehrlich reaction as an indication of the presence of melanogen must be verified by concentrating the urine and then demonstrating a positive Thormählen test. In any case, the positivity of both reactions indicates the presence of melanogen with absolute certainty.

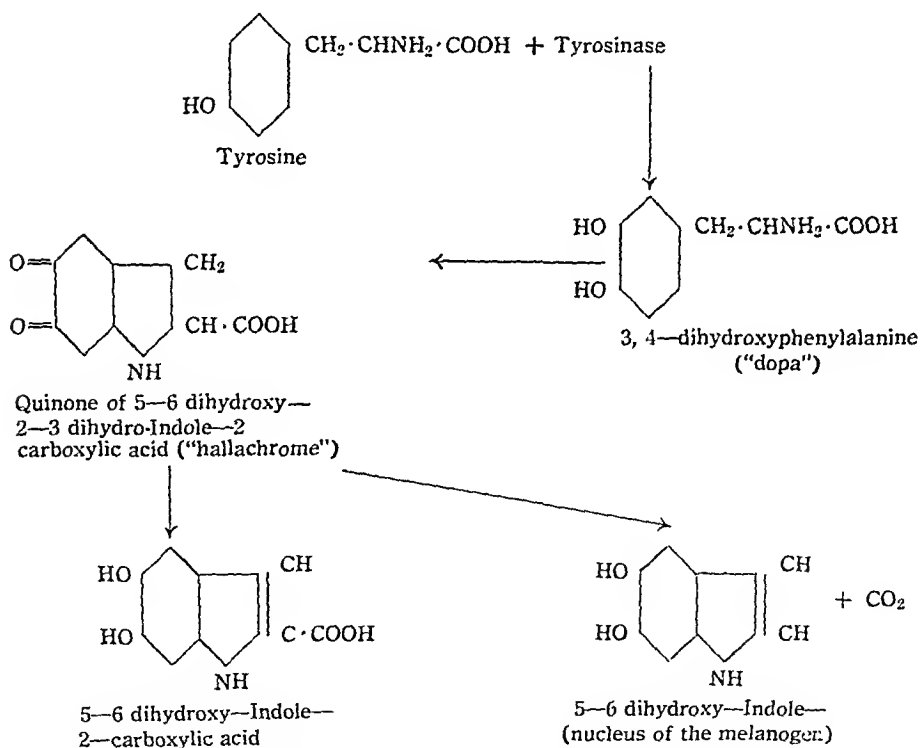


Fig. 1.—Formation of melanin precursors (Raper').

It has been known for a long time that Thormählen's reaction is given only by indole and its derivatives. However, the origin of the indole compounds in the melanotic urine was not understood until Raper<sup>4</sup> in his fundamental work traced step by step the chemistry of melanin formation from tyrosine. Before that it was an accepted assumption that the source of the "Thormählen substance" is tryptophan as the only natural indole compound in the protoplasm.<sup>5</sup>

Raper presented evidence that indole derivatives are formed from tyrosine in the course of melanin formation, and it seems today that the only source of melanin pigment in the whole animal world is tyrosine.

In the course of oxidation of tyrosine to melanin by tyrosinase<sup>4</sup> or by ultra-violet light<sup>5, 7</sup> the first step is the formation of dihydroxyphenylalanine, or by abbreviation "dopa" (Fig. 1). The next step is the formation of a ring by the alanin side chain so that an indole nucleus results which is a condensed benzene and pyrrole ring. In 1935 Linnel and Raper<sup>3</sup> presented strong supporting evidence that the melanogen is the monopotassium salt of 5-6 dihydroxy-indole. In acknowledging the findings of Raper it became easy to understand that in addition to the indole derivatives, catechol compounds also (derivatives of dopa) were found as melanin precursors in melanotic urine.<sup>8, 9</sup>

#### EXPERIMENTAL

Most experiments were carried out with the urine of a 67-year-old patient from the service of Dr. George F. Diek. The patient presented the usual history of persons suffering from malignant melanomas. His left eye had been enucleated one year ago. He was admitted, very ill with jaundice and liver enlargement. His urine had been dark for several weeks. Only about three liters of this patient's urine could be collected since he was suffering from incontinence, and the daily urine obtained averaged 300 c.c. daily for ten days, after which time he died. Permission for autopsy was not obtained.

One urine specimen of another patient with a similar history was supplied by Dr. M. H. Ebert from the Rush Medical School, Chicago. This specimen amounted to only 70 c.c.

The material was first used for estimating the sensitivity of the different melanogen tests. This estimation was carried out by serial dilution of the urine with water and estimation of the threshold dilutions at which the reactions became negative. As mentioned in the introductory remarks, it was found that Thormählen's test was 50 times, and the Ehrlich test was 100 times, as sensitive as the ferric chloride test.

The material was used for answering two other clinically important questions: (1) Whether by simple methods the melanogen can be differentiated from indole and indican; and (2) whether the melanogen can be concentrated by fractionation of the urine so that smaller amounts may be detected in the fraction than is possible in the whole urine.

1. The qualitative chemical differentiation of the melanogen, indole, and indican was possible by applying the common laboratory tests for indole derivatives. As shown in Table I it was found that urine containing large amounts of indican does not give the Thormählen, Ehrlich, and Herter indole reactions, and vice versa. The melanogen does not react with Obermayer's and with Jolles' reagents. Indole itself displays the same color reactions as the melanogen, but with one remarkable exception: Herter's reaction most sensitive for indole is negative in the melanotic urine. This is an expedient to differentiate indole from the melanogen.

2. Concentration of melanotic urine for clinical purposes was carried out by Blackburn and Wanger,<sup>1</sup> but their method was based on the oxidation of

melanogen to melanin and not on the sensitive color reactions of indole. Fractionations for purposes of chemical identification of the melanogen were made by several authors.<sup>3, 9, 10</sup>

Testing all recommended methods of fractionation we found that the simplest and most effective method was precipitation with lead acetate.

TABLE I

QUALITATIVE COLOR TESTS OF INDOLE AND ITS DERIVATIVES OCCURRING IN THE URINE

| TEST                         | INDOLE | INDICAN | MELANOGEN |
|------------------------------|--------|---------|-----------|
| Herter's naphthoquinone test | +      | -       | -         |
| Ehrlich's aldehyde test      | +      | -       | +         |
| Thormählen's test            | +      | -       | +         |
| Nitroso-indole test          | +      | -       | +         |
| Obermayer's test             | -      | +       | -         |
| Jolles' test                 | -      | +       | -         |

The fresh urine was acidified with acetic acid to a pH of about 4.5, and lead acetate was added. The precipitate containing phosphates and sulfates was discarded. The clear filtrate, then, contained the total amount of melanogen. If this filtrate was alkalinized with ammonia to about pH 8, the newly formed lead precipitate took down most of the melanogen. After sharp centrifugation and separation from the supernatant fluid, the precipitate was ground up in small amounts of water. The suspension was decomposed by introduction of hydrogen sulfide. The precipitated lead sulfide was removed by filtration; the excess hydrogen sulfide by cautious heating. The clear yellow filtrate contained three to ten times as much melanogen as the original urine had in the same volume.

## ADDITIONAL OBSERVATIONS

1. In the fractions obtained by precipitation with lead acetate the melanogen proved to be much more stable than in the original urine. Positive Thormählen and Ehrlich reactions could be demonstrated in these fractions after the samples were refrigerated in the dark for four months, covered with a cork stopper only, unprotected against oxidation. Obviously this stability of the melanogen was not due to the acidity of the fractions (pH 5) because an even better protection of the melanogen was obtained with barium hydroxide in slightly alkaline medium.<sup>10</sup> It seems rather that any kind of fractionation, consisting of rough chemical procedures, removes or inactivates a component of the urine which accelerates the oxidation of melanogen to melanin. It may be assumed that this factor is the enzyme of melanin formation which is present in pigmentary malignant tumors<sup>11, 12</sup> and which could be spilled over in the urine together with the melanogen.

2. Dr. F. K. Oldham, of the Department of Pharmacology, using hypophysectomized frogs as test objects, demonstrated melanophore activity in our melanotic urine samples. Earlier experiments with similar results were carried out on animals which were not hypophysectomized.<sup>13, 14</sup> However, this assay is reliable on hypophysectomized frogs or fishes only.<sup>15</sup> Roughly concentrated fractions of the melanotic urine had no darkening effect on the hypophysectomized frog.



3. The originally light yellowish acid urine fractions with high melanogen content turned brilliant red on standing in the icebox. Similar observations were made as early as 1890,<sup>16</sup> and a contemporary thorough chemical study of the red substance in melanotic urine was made in 1936.<sup>9</sup>

Chemical and spectrographic analysis of the red pigment in our laboratory showed that it is similar to the acid-soluble red pigment of human hair, first described by Arnow in 1938,<sup>17</sup> but is not identical with it. The detailed results of these experiments will be described elsewhere.

# DISCUSSION

The fact that melanogen does not show indican reactions and that indican does not give positive indole reactions is in accord with the statement of Böhm.<sup>18</sup> In 1939 Böhm, in a different way, reached the conclusion that indigo blue formation, on which the indican tests are based, occurs only in those indole derivatives which have substituted C atoms on the pyrrole ring so that they can react there. The 5-6 dihydroxy-indole compound of Raper can react on the fifth and sixth C atom of the benzene ring but not on the pyrrole ring, and, therefore, does not yield indigo blue on oxidation (Fig. 2).

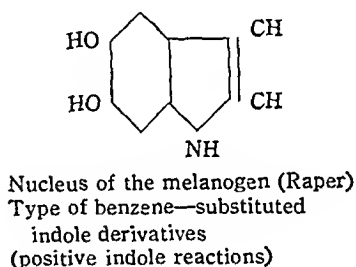
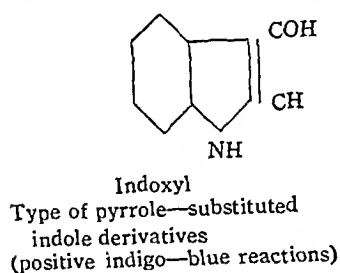


Fig. 2.

The unexpectedly negative Herter reaction of the melanogen which we obtained has not been previously reported.

By the concentration method described above, the test for melanuria has been rendered up to ten times as sensitive as is the Thormählen test and up to 500 times as sensitive as is the ferrie ehloride test in the original urine. It seems probable that the diagnosis of melanuria can be made considerably earlier in this way.

It has been elaimed<sup>1, 5</sup> that the urine becomes dark only after the liver has been involved to a considerable degree in the metastatic process so that a functional insufficiency of the liver results. If this were true, if the melanuria indicated marked liver damage rather than growth of melanotic tumors anywhere in the body, there would be little hope of discovering the melanuria in an early state by concentration methods. However, there is strong evidencee against this theory. By feeding indole derivatives which are not substituted on the pyrrole ring to normal animals and normal human beings, melanuria can be easily provoked, which is chemically identical with pathologic melanuria following malignant pigmentary growth.<sup>18</sup> Thus melanuria can be provoked in normal organisms without actual liver damage. It seems that melanuria depends only on the amount of melanogen present in the body. If this amount exceeds the threshold, either as a result of feeding or as a result of the metabolic process

in the tumor, melanogen is excreted through the kidneys. There is no reason to assume that exceeding of the threshold occurs suddenly with spilling over of large amounts of melanogen from the beginning. It is more probable that there is a gradual overstepping of the threshold similar to the case of glycosuria or indicanuria. In this case "latent melanuria" may precede macroscopically obvious melanuria by weeks or months. Therefore, in patients who are thought to have melanotic tumors, the *concentration method* as described above might be useful for an early diagnosis.

#### SUMMARY

1. The melanogen of melanotic urine can be differentiated from other indole compounds occurring in the urine by simple qualitative chemical tests.
2. By the use of Thormählen's and Ehrlich's reactions in a urine suitably concentrated, the test for melanuria can be rendered 500 times as sensitive as it is with the ferric chloride test in the original urine.
3. By rough chemical fractionation the melanogen becomes more stable than it was in the original urine.
4. The melanotic urine has melanophore activity which disappears after rough chemical treatment.
5. The red pigment formed in melanotic urine after acidification and the pigment of human red hair have similar properties.

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## A RAPID METHOD FOR THE DETERMINATION OF SERUM PROTEIN\*

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MANY indirect methods, designed to replace the time-consuming Kjeldahl procedure, have been proposed for the determination of serum protein. These have been reviewed by Peters and Van Slyke,<sup>1</sup> who conclude that the specific gravity method is the most reliable. This makes use of the linear relationship which exists between the specific gravity of the plasma or serum and the total protein content. The existence of such a relationship for human plasma was first demonstrated by Moore and Van Slyke.<sup>2</sup> Weech, Reeves, and Goettsch<sup>3</sup> then used dog's serum and found an even closer correlation. Kagan<sup>4</sup> examined specimens of human serum and derived an empirical relationship differing only slightly from those previously reported.

For determining the specific gravity, Moore and Van Slyke used a small pycnometer and an analytical balance. The falling-drop technique of Barbour and Hamilton,<sup>5</sup> developed previously, offers the advantage of being rapid as well as accurate; but the apparatus is relatively complex, and considerable experimental skill is required for its operation. Bing<sup>6</sup> described a method in which serum is diluted with measured amounts of 0.9 per cent sodium chloride until it acquires a specific gravity corresponding to 3.0 per cent protein, as indicated by an accurately adjusted float in the form of a glass bead. Instead of a single bead Simeone and Sarris<sup>7</sup> employed twenty-one glass beads made at random in large numbers and carefully selected according to their densities so as to form a graded series covering the range of serum protein values. In use, these beads were immersed in the serum, one at a time, until a bead was found with a specific gravity approximately equal to that of the serum.

The present paper describes a specific gravity method for the determination of serum protein which is both rapid and accurate. The essential apparatus consists of a single article of glassware of relatively small dimensions which can be calibrated to read directly in either specific gravity or protein content. Only ordinary glass-blowing skill is required for its construction.

### METHOD

The apparatus consists of a specially constructed pipette (Fig. 1b), containing two hollow glass heads. One bead is slightly lighter and the other is slightly heavier than the range of sera to be examined. In use, the pipette is filled with serum and placed in an upright position, the beads coming to rest at points *k* and *k'*. It is then closed and inverted by rotating 180 degrees.

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After inversion the lighter bead will move upward while the heavier one descends, the ensuing collision occurring at a point which is determined primarily by the specific gravity of the liquid contained in the pipette. By observing the position of this point with reference to a properly calibrated scale engraved on the tube (Fig. 1*d*), the specific gravity of any sample falling within the range covered by the beads can be found.

In filling the pipette it is convenient to use a suction tube, into which a snugly fitting solid glass bead has been inserted. Using the bead as a valve in the usual way, the liquid can, with ease, be drawn up into the pipette and retained while a rubber band is stretched over the tip. The suction tube is then removed and the closure is completed after the manner of blood pipettes. The trap, *t*, is designed to catch air bubbles. The form shown in Fig. 1*a* does not require a rubber band, and air bubbles, if present, are removed through one of the stopcocks. In use, this type of pipette is not filled completely; a small amount of air is left in the capillary below the upper stopcock in order to permit expansion of the liquid. The capillary must have sufficient length to serve this purpose.

Rotation of the pipette is facilitated by use of a holder, arranged so that it may be turned through 180 degrees in a vertical plane. This consists of a light metal frame provided with a spring clip for holding the pipette, and is mounted on a horizontal axle, which in turn is supported on a base equipped with leveling screws. Movement of the frame is limited to 180 degrees by means of stops. For ordinary measurements, however, satisfactory results are obtained simply by inverting the pipette while it is held in the hands.

No particular size of pipette is required. Capacities of 1 or 2 c.c. have been found to be satisfactory. The bulb is made of standard wall tubing having an internal diameter of about 4 mm. and a length of 80 mm. in the case of the 1 c.c. pipette, and 5 mm. and 100 mm. for the larger size. The capillary stems have a bore of approximately 1 mm., with the exception of the portion immediately below the trap, which is blown out to about twice this size in order to permit freer passage of air bubbles into the trap. The diameter of the beads is somewhat less than one-half that of the tube in which they are enclosed. It is well to have them as nearly equal in size as possible. This is accomplished by preparing a number of beads and matching them according to size with the aid of a low-power microscope provided with a micrometer eyepiece.

The beads are blown from thin-walled, capillary, pyrex tubing. The desired density is obtained by collecting a small lump of glass on one end and grinding off a little at a time until the bead neither sinks nor rises rapidly when placed in a solution of potassium sulfate of predetermined specific gravity. It is then returned to the flame where the remaining portion of the lump is fused into the main body of the bead. When made in this way, a bead will, in general, have a thick wall at one end; but this is not detrimental to its usefulness. Final adjustment, if necessary, may be made by grinding the thick end. The ground area is fire-polished in a small flame at a relatively low temperature. This procedure not only gives a much higher yield of useful beads than mere random methods, but also results in a considerable saving in time.

The walls of the tube are indented at  $k$  and  $k'$  by heating the glass in spots 120 degrees apart, and pushing inward with a thin brass tool. These indentations provide seats in which the heads are brought to rest in the center of the tube. Considerable care is required in making them so as to reduce the area of contact to a minimum, and also to allow sufficient space for the liquid to pass freely around the bead at the beginning of an excursion.

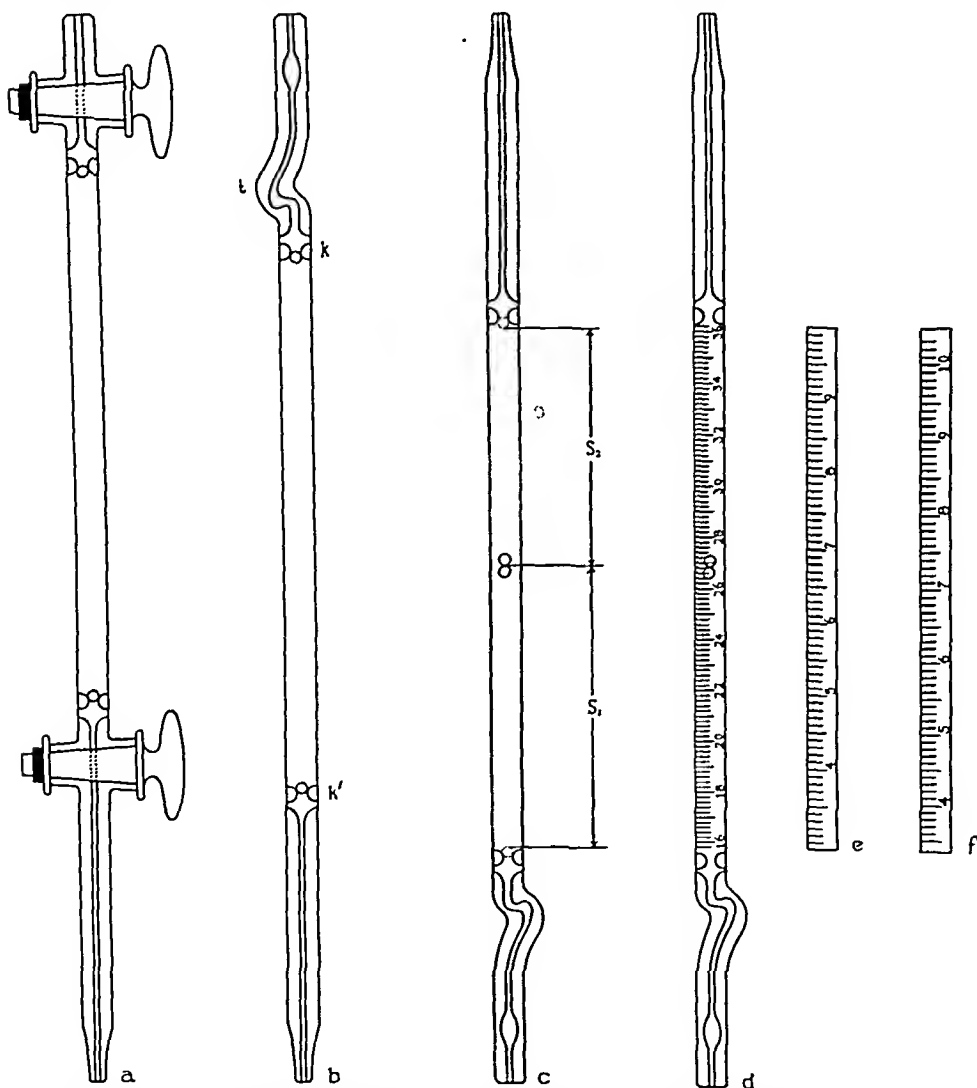


Fig. 1.—Specific gravity pipettes. *a*, With stopcocks; *b*, for rubber band closure; *c*, inverted, showing displacement of glass beads; *d*, graduated to read in specific gravity, figures representing second and third decimal places. Reading shown is 1.0268, corresponding to a serum protein content of 6.8 per cent. *e*, Scale for pipette (*d*) reading directly in percentage of serum protein; *f*, direct reading protein scale constructed from calibration curve in Fig. 2,  $K' = 0.96$ .

#### THEORY

When a small sphere falls under the action of gravity through a viscous fluid, the resistance increases with increasing rate of fall until it is equal and

opposite to the effect of gravity. Thenceforth, it has a constant velocity, for there is no resultant force acting upon it. This velocity is given by the well-known Stokes' law as

$$v = \frac{2g \alpha^2 (d-D)}{9\eta} \quad (1)$$

where  $d$  is the density of the sphere,  $D$  the density of the fluid,  $\eta$  the coefficient of viscosity of the fluid, and  $\alpha$  the radius of the sphere.

Stokes' law as given applies only to a spherical body falling through a fluid of infinite extension, but the velocity of a small sphere falling axially through a viscous liquid in a cylindrical tube may be expressed by the modified form

$$v = \frac{K (d-D)}{\eta} \quad (2)$$

$K$  being a numerical constant for a particular tube. Then, replacing the velocity by its equivalent  $S/t$ , where  $S$  represents the distance traversed in the time,  $t$ , and applying the resulting formula to the motion of each bead in turn, two equations are obtained which may be combined to give the relation

$$\frac{S_1}{S_2} = K' \frac{D-d_1}{d_2-D} \quad (3)$$

where  $S_1$  and  $S_2$  are the distances traveled at constant velocity before collision by the light and heavy bead, respectively, and  $d_1$  and  $d_2$  are their densities. The value of  $K'$  is constant for a given apparatus, and is equal to unity when the two beads have the same effective radius. Since both beads are accelerated at the start in the same manner and reach their limiting velocities within a comparatively short distance,  $S_1$  and  $S_2$  may be taken to represent the entire distances traversed without introducing significant error.

An exact expression should take account of the repellent force exerted by one bead upon the other as the point of collision is approached. It can be shown that both are affected by this factor to the same extent only when their velocities are equal. Another factor is the "end effect," i.e., the effect of the ends of the tube on the velocity. The complete analysis, however, presents an involved problem in hydrodynamics.

Although formula (3) is obtained by an approximate treatment, it is sufficiently accurate to be used for purposes of calibration. It was tested in an extended series of experiments using matched beads of various sizes and densities in solutions of potassium sulfate as well as in serum, and found to hold, within rather narrow limits, over a wide range of fluid specific gravities. Typical results are shown in Fig. 2. It will be seen that the greatest deviations occur in the case of extreme values. None is of sufficient magnitude, however, to be of practical importance. It may be said that the accuracy with which the equation fits experimental values justifies the assumptions used in its derivation.

#### CALIBRATION

If the densities of the beads are known, a pipette may be calibrated by taking a single collision point reading on serum or a salt solution of known specific gravity. The reading should fall near the midpoint of the bulb, since

in this case errors of reading and inaccuracies in the formula will have a minimum effect. The beads are most conveniently calibrated, before being sealed in, by matching them with solutions of potassium sulfate and subsequently determining the specific gravities of the solutions with a pycnometer. With  $d_1$  and  $d_2$  thus found together with values for  $S_1$  and  $S_2$ , the constant,  $K$ , in equation (3) is computed. Having evaluated  $K$ , it is possible to calculate the distances,  $S_1$  and  $S_2$ , for any specific gravity lying within the range of the beads.

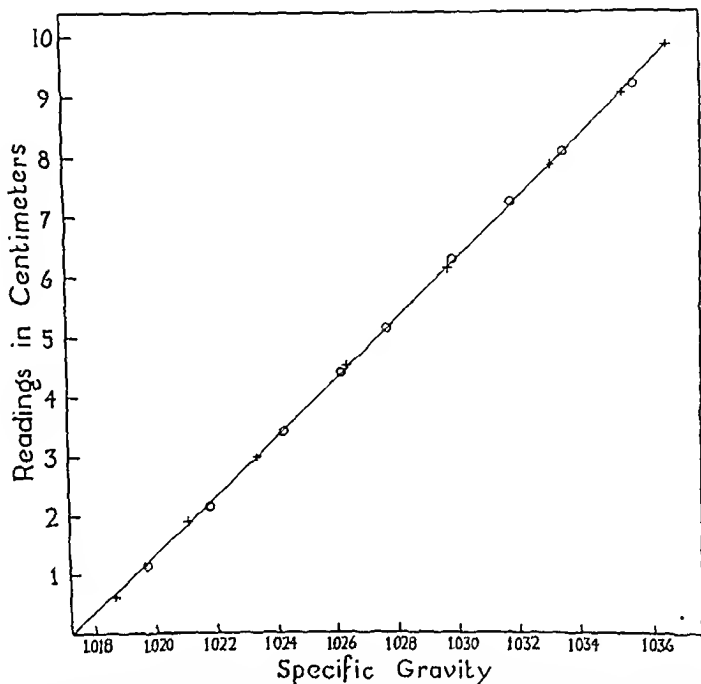


Fig. 2.—Typical calibration curve. Readings represent distance,  $S_1$ , traversed by light bead. Curve was drawn from points computed by equation (3) from bead densities and one collision point reading. Crosses indicate solutions of potassium sulfate; circles denote serum modified by dilution or evaporation.

A more direct method of calibration may be used in which collision point readings are taken on a series of standard solutions. Each point is marked with ink or a sharp wax pencil, and its distance,  $S_1$ , from the end of the bulb is measured by direct comparison with a millimeter scale. The specific gravities of the solutions are then plotted accurately on coordinate paper against the readings,  $S_1$ , and a smooth curve is drawn through the points. For ordinary work a curve based on four or five well-spaced points should suffice.

The two methods are compared in Fig. 2. It may be pointed out that the first method does not require the drawing of a curve. Regardless of which method is followed, a scale is readily constructed to read directly in specific gravity (Fig. 1*d*). Calibration in terms of percentage of protein is made with the aid of the empirical formula of Weech, and co-workers.<sup>3</sup> Integral protein percentages are substituted in the formula, and the corresponding specific gravities are calculated. The latter may be used in either of the foregoing methods for preparing a direct reading scale such as shown in Fig. 1*e*.

## TEMPERATURE CORRECTION

In order to determine the influence of temperature, a pipette calibrated at 20° C. was filled with human serum and placed in a thermostatically controlled water bath so arranged that any predetermined temperature could be maintained within one-twentieth degree. Specific gravity readings were taken at intervals of five degrees, from 15° to 35°, with results as shown in Table I. Within the limits of experimental error the readings follow the thermal expansion of serum, indicating that the change in density of the serum is the only important factor involved. The expansion of the pyrex glass beads is about one-twenty-fifth that of serum and may be disregarded in dealing with a relatively narrow temperature range.

TABLE I  
EFFECT OF TEMPERATURE ON PIPETTE READING

| TEMPERATURE, °C. | SPECIFIC GRAVITY | PROTEIN<br>(GRAMS PER 100 C.C.) |
|------------------|------------------|---------------------------------|
| 15               | 1.0310           | 8.3                             |
| 20               | 1.0300           | 7.9                             |
| 25               | 1.0288           | 7.5                             |
| 30               | 1.0274           | 7.0                             |
| 35               | 1.0258           | 6.4                             |

It follows that a pipette which is calibrated at 20° C. and used at a temperature,  $t^\circ$ , will read in specific gravity at  $t^\circ/20^\circ$  C. Between 20° and 30° the change in serum specific gravity averages about 0.00025 per degree centigrade. A reading taken within this temperature range may be reduced to specific gravity 20°/20° C. by adding 0.00025 to the observed specific gravity for each degree centigrade above 20. Such correction is accurate within one unit in the fourth decimal place. For clinical purposes an approximate correction may be applied by adding 0.1 to the percentage of protein for each degree rise in temperature.

## RESULTS

In order to test the accuracy as well as the clinical practicability of the method, measurements were carried out on a large number of serum specimens, and the results were compared with those obtained by the pyknometer and falling-drop methods. A 2 c.c. pyknometer of the Nicol type provided with a graduated stem was used. This was found to be superior to the specific gravity bottle of Moore and Van Slyke<sup>2</sup> in accuracy, and ease of filling and cleaning. The falling-drop method followed the technique described by Barbour and Hamilton.<sup>5</sup> The blood samples were obtained by venepuncture from patients selected in most cases because they were known or believed to have abnormalities in their serum proteins. Typical results are summarized in Table II. Total protein estimations by the macro-Kjeldahl method are included for comparison.

Undoubtedly the pyknometer method is the most reliable one available for the determination of specific gravity. The average difference between the results obtained by the pyknometer and the specific gravity pipette is less than 0.0002. In the case of the falling-drop method the average difference is substantially the same, indicating that the method described here compares favorably in accuracy with the falling-drop technique.



The results obtained in the case of specimen No. 4 are of special interest. Repeated trials by the falling-drop method gave low values with a rather large variation among the individual determinations. This specimen was characterized by a cholesterol content of over 850 mg. per 100 c.c., 33 per cent of which was present in the form of the ester. Whether or not the failure to obtain satisfactory results can be ascribed to the unusually high content of these constituents was not ascertained. However, no difficulty was experienced with the specific gravity pipette.

TABLE II

COMPARISON OF RESULTS OBTAINED BY THE SPECIFIC GRAVITY PIPETTE WITH THOSE OBTAINED BY OTHER METHODS

| SPECIMEN | SPECIFIC GRAVITY |              |         | PROTEIN (GRAMS PER 100 C.C.) |          |
|----------|------------------|--------------|---------|------------------------------|----------|
|          | PYKNOMETER       | FALLING DROP | PIPETTE | PIPETTE                      | KJELDAHL |
| 1        | 1.0187           | 1.0188       | 1.0185  | 3.90                         | 3.97     |
| 2        | 1.0203           | 1.0204       | 1.0204  | 4.57                         | 4.61     |
| 3        | 1.0203           | 1.0203       | 1.0205  | 4.61                         | 4.73     |
| 4        | 1.0207           | 1.0198       | 1.0207  | 4.68                         | 4.96     |
| 5        | 1.0222           | 1.0222       | 1.0223  | 5.23                         | 5.50     |
| 6        | 1.0234           | 1.0232       | 1.0233  | 5.58                         | 5.68     |
| 7        | 1.0261           | 1.0260       | 1.0263  | 6.63                         | 6.62     |
| 8        | 1.0286           | 1.0284       | 1.0285  | 7.40                         | 7.43     |
| 9        | 1.0311           | 1.0312       | 1.0311  | 8.30                         | 8.21     |
| 10       | 1.0333           | 1.0330       | 1.0336  | 9.18                         | 9.01     |

## DISCUSSION

Once the apparatus has been constructed and calibrated, the determination of serum specific gravity and total protein content becomes a matter of extreme simplicity. In making a determination it is necessary merely to fill the pipette, stretch a rubber band over the ends, invert it, and take the reading. In fact, a fair degree of accuracy can be obtained simply by inverting the pipette while it is attached to a rubber suction tube provided with a glass bead valve, thus dispensing with the use of a rubber band. For the greatest accuracy the use of the rotating holder is advised.

The pipette is cleaned and dried quickly by drawing water through it, followed by alcohol, ether, and air.

It is important to know the temperature at which a measurement is made. Ordinarily the reading of a thermometer placed near the apparatus is sufficient. In order to avoid warming the liquid, the pipette should be handled only by the capillary stems at points well removed from the bulb.

Certain other precautions should also be observed. Rotation must be carried out at such a rate that the beads will be kept in place by centrifugal force and yet not be thrown out of position as the inverted pipette is brought to rest. On account of the viscosity of serum this operation is not particularly critical and can be learned by a few trials. The most serious source of error arises from small air bubbles which adhere to the glass beads. These are readily seen in good light and when present must be shaken off. Difficulty from this source rarely occurs with serum used soon after centrifuging. It is most likely to occur with refrigerated serum, particularly if it is drawn into the pipette before coming to the temperature of the room.

Obviously, the method described here may be used to determine the specific gravity of any body fluid which is not opaque, if 1 c.c. or more is available. Various ranges may be covered by preparing glass beads of the required specific gravity. Furthermore, preliminary work has shown that the albumin and globulin fractions of serum may be determined separately by a modification of this method.

#### SUMMARY

1. A new specific gravity method for the determination of serum protein has been developed.
2. The method is rapid and accurate, and has the advantage of being direct reading.
3. The essential apparatus consists of a single article of glassware, the construction, calibration, and use of which are described in detail.

I am indebted to Dr. Newton Evans, of the Los Angeles County General Hospital, for his assistance in obtaining many serum specimens used in this work.

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# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**TISSUE:** Modification of Mallory's Phosphotungstic Acid-Hematoxylin Stain for Formaldehyde-Fixed Tissues, Peers, J. H. Arch. Path. 32: 446, 1941.

1. Deparaffinize sections and bring down to water.
2. Mordant three hours in a saturated aqueous solution of mercuric chloride in a paraffin oven at 57° C. Rinse briefly. The mercuric chloride solution may be used repeatedly.
3. Place in compound solution of iodine for five minutes. Rinse.
4. Place in 5 per cent aqueous solution of sodium thiosulfate for five minutes. Rinse.
5. Place in 0.25 per cent potassium permanganate for five minutes. Rinse.
6. Place in 5 per cent aqueous oxalic acid for five minutes. Wash well.
7. Stain overnight in phosphotungstic acid hematoxylin.
8. Wash briefly in tap water, dehydrate in alcohol or acetone, clear in xylol, and mount in balsam.

This technique gives results almost identical with those of the original method as applied to tissue fixed in Zenker's solution. Neuroglia, fibroglia, and myoglia fibrils, blepharoplasts, nuclei, red blood cells, and fibrin are deep blue, while collagen is brownish red.

**TULAREMIA,** Lawless, T. K. Arch. Dermat. & Syph. 44: 147, 1941.

The following antigen is proposed:

The pus from an unopened gland is aspirated and divided into three equal portions. These separate portions are triturated with ten volumes of acetone, alcohol, and 0.4 per cent saline solutions. The mixture is allowed to digest for twenty-four hours, with frequent agitation. The supernatant fluids are pipetted off and mixed. The alcohol and acetone are allowed to evaporate, and the original volume is made up with 0.4 per cent saline solution. The solution is sterilized on a water bath at 60° C. for two hours and twenty-four hours later for one hour. Tests for sterility are performed on agar plates. Phenol, 0.25 per cent, is then added.

As a test material, 0.1 c.c. is introduced intradermally, and the reaction is read at the end of one-half hour. A large erythematous, edematous wheal with pseudopodia in some cases will result in a case of the disease. This reaction remains for twenty-four or more hours, depending on the severity of the condition. A control subject should show either no reaction or one that disappears in ten to fifteen minutes. In patients presenting non-specific reactions, the wheal disappears in from one to two hours. The earliest time at which a positive result was obtained was one day after onset of symptoms. Two weeks later a positive reaction was obtained with a tularemia agglutination test with a 1:1,280 dilution. The oldest condition was recognized five months after infection and had been diagnosed and treated as influenza, pneumonia, and empyema.

This antigen also agglutinates the red blood cells of infected persons and produces a specific histiocytic response in the local tissues.

**PLASMA PROTEINS,** Simple Method for Estimating, Walther, W. W. Lancet 241: 337, 1941.

**Materials:** Pyrex boiling tubes, 170 mm. by 28 mm., marked by a ring at 25 cm.  
"Antibump" device consisting of 5 mm. of glass tubing fused to the end of a glass rod 160 mm. long.

Pure, nitrogen-free sulfuric acid.

Pure, nitrogen-free potassium persulfate saturated solution.

Nessler's solution (special for blood testing).

Nitrogen standard. Pure ammonium sulfate, 0.03776 Gm. per liter of distilled water containing 7 c.c. of pure sulfuric acid. It is convenient to prepare this by diluting 8 c.c. of a 0.472 per cent solution of the salt to 1 liter.

Analyses may be done on heparinized plasma (using the minimum of heparin) or on serum.

*Method:* After washing out the boiling tube with distilled water, about 0.5 c.c. of water collects in it. Using the hemoglobin pipette, wash out 20 c.c. of serum or plasma into this water. Add 0.25 c.c. of pure sulfuric acid and mix. Boil gently over by-pass flame of Bunsen burner with "antibump" rod in position. When thick white fumes rise and fill the tube, cover with a watch glass and boil for three minutes. The solution is then quite black. Remove the flame for two minutes, and then add 0.5 c.c. of saturated potassium persulfate solution. Mix with antibump rod and replace flame. Boil without watch glass until thick white fumes rise again, then replace the watch glass and boil for two minutes longer, after which the solution will be clear and colorless. Cool, dilute with distilled water to the 25 c.c. mark, holding the tube with the 25 c.c. mark at eye level and remove the antibump rod. Replace the rod and wash well in the solution. Use any convenient volume (the author uses 5 c.c. of the solution placed in an ordinary test tube), and add 2 c.c. of Nessler's solution. A similar volume of standard solution is treated in the same way. Mix by inverting twice and compare at once in the colorimeter. Equal color indicates 1,000 mg. of total nitrogen per 100 c.c. of sample. A direct reading may be obtained by fixing the unknown at 10 and moving the standard to match. The reading of standard times 100 equals milligrams of total nitrogen per 100 c.c. of sample. The reading is greatly facilitated by placing a spectrum blue color filter over the eyepiece. Unless gross nitrogen retention is suspected, a subtraction of 50 mg. to cover the nonprotein nitrogen gives the figure for protein nitrogen which, when multiplied by 6.25/1,000 gives the protein percentage. The reagents should be tested occasionally by blank estimations.

If a colorimeter is not available, a comparator or similar instrument can be used, but the reading of the yellow Nessler solution is greatly helped by using a blue light filter. The accuracy of the method depends on individual skill in color matching, but it is well within the limits necessary for clinical work.

#### DIPHTHERIA, Present Status of the Immediate Tellurite Test for, Bierman, H. R., and Maxwell, R. W. J. A. M. A. 117: 1255, 1941.

The potassium tellurite procedure for the differential diagnosis of diphtheria, as suggested by Manzullo, was applied to 117 patients exhibiting membranes of the throat.

The test was frequently positive on nondiphtheritic membranes but was falsely negative only twice.

A negative tellurite reaction, if it contradicts the clinical impression, is not sufficiently reliable to justify one in withholding antitoxin therapy.

It is felt that the technical difficulties of applying the test at present, and the frequency with which both false positive and false negative reactions are obtained, make it unsatisfactory as a routine guide for the therapy of diphtheria. Refinements in technique might admittedly enhance its usefulness.

#### SYNOVIAL FLUID and Synovial Membrane Abnormalities Resulting From Varying Grades of Systemic Infection and Edema, Coggeshall, H. C., Bennett, G. A., Warren, C. F., and Bauer, W. Am. J. Med. Sc. 202: 486, 1941.

From a study of 156 post-mortem synovial fluids and 49 synovial tissue specimens obtained from persons dying with varying degrees of infection and edema, the following conclusions can be drawn:

1. The amount of synovial fluid in the knee joints of persons with peripheral edema is usually increased.

2. The synovial fluid obtained from the joints of edematous patients contains fewer nucleated cells than does normal fluid and its total solids, viscosity, protein, and mucin contents are reduced.

3. Total nucleated cell counts and the absolute number of neutrophils in synovial fluid specimens obtained from patients dying with varying degrees of infection are frequently increased. The most marked cytologic abnormalities are observed in patients dying with severe infections and associated septicemias.

4. An increase in the absolute number of neutrophils is a more reliable indication of the existence of synovial tissue inflammation than are increased total nucleated cell counts or random biopsies.

5. The synovial fluid abnormalities resulting from synovial tissue infections vary with the severity of the synovial lesion and its proximity to the joint space.

6. The results of the present study suggest that the joint aches and pains occurring in various infectious diseases may be the result of synovial tissue inflammatory changes caused by blood-borne infections. The majority of such inflammatory lesions are presumably mild in nature and, therefore, resolve readily. The marked synovial tissue reactions observed in patients dying with septicemia are of the type observed in early cases of specific infectious or septic arthritis.

**TRANSFUSIONS, Infusions of Blood and Other Fluids Via the Bone Marrow, Tocantins, L. M., O'Neill, J. F., and Jones, H. W. J. A. M. A. 117: 1229, 1941.**

Citrated blood and physiologic solution of sodium chloride have been infused through the marrow of a tibia or a femur of 9 infants in whose treatment intravenous infusions were urgent but impossible. In 2 with congenital anemia and erythroblastosis, it was not possible to infuse any blood by this method. No local or constitutional reactions, immediate or delayed, have been disclosed by clinical and roentgen-ray examinations after these infusions. When clearly indicated, the intramedullary route for parenteral therapy is feasible and useful.

**BLOOD IODINE: Naturally Occurring Iodine Fractions and Their Chemical Behavior, Bassett, A. M., Coons, A. H., and Salter, W. T. Am. J. Med. Sc. 202: 516, 1941.**

The protein-bound iodine in the blood plasma of man and of animals was found to reside chiefly in the traditional albumin fraction. This iodine was subject to fluctuations dependent upon thyroid activity. Such fluctuations, in absolute terms, were due chiefly to a thyroxine-like moiety thereof. The moiety resembling diiodotyrosine might increase proportionately, but it contributed relatively little to the absolute change from the normal level. Accordingly, total protein-bound iodine fluctuated largely with thyroxine-like iodine. In myxedema the thyroxine-like fraction practically disappeared.

Despite variations in this protein-bound iodine, the ionized iodine (inorganic) appeared to be rather low and approximately constant under the conditions of this study, regardless of the state of thyroid activity. Nevertheless, it was found to increase markedly when extraordinary, though perhaps very small, amounts of iodine entered the organism. Simultaneously a false increase in the protein-bound iodine occurred which could be reproduced by the addition of iodide to plasma *in vitro*.

The results indicate that the protein-bound thyroxine-like moiety of the plasma iodine is a good objective index of circulating thyroid hormone. They suggest, also, that plasma protein-bound iodine might be used clinically to confirm physiologic thyroid status.

**BLOOD IODINE: Its Relation to Thyroid Function in 100 Clinical Cases, Salter, W. T., Bassett, A. M., and Sappington, T. S.**

A series of 100 cases of suspected thyroid disturbance and 10 control patients have been analyzed from the standpoint of final clinical diagnosis, basal metabolic rate, and plasma protein-bound iodine. When the first two of these criteria are in agreement, there is a close correlation between the plasma iodine and the basal metabolic rate. Such cases amount to 71 per cent of the entire group studied. Of the remaining 29 per cent, the basal metabolic rate often did not clearly reflect the clinical status and the plasma protein-bound

iodine proved much more reliable. This was particularly true in the group of "Graves' without hyperthyroidism" in which the basal metabolic rate ranged from -20 to 40 per cent, but the plasma protein-bound iodine was normal. These data, therefore, constitute additional evidence for the possible dissociation of physiologic hyperthyroidism and clinical Graves' disease. In hypothyroidism plasma protein-bound iodine appears to be a highly reliable criterion for confirming lack of thyroid hormone, even when full-blown myxedema is absent.

It has been emphasized that the simplified chemical procedures employed are partly empirical. Their use has been justified by their consistency with clinical data, but due care should be exercised in comparing the data with the results obtained by other methods.

**BLOOD: The Use of Rabbit Thrombin as a Local Hemostatic, Lozner, E. L., MacDonald, H., Finland, M., and Taylor, F. H. L. Am. J. Med. Sc. 202: 593, 1941.**

Observations are reported indicating that rabbit thrombin is of distinct value in the control of hemorrhage from small wounds induced by a standard trauma in normal persons and in 9 patients with and in 2 patients without hemorrhagic diatheses bleeding spontaneously from small wounds.

The material apparently accomplishes its results by the instantaneous precipitation of fibrin.

No toxic manifestations were observed following the application of rabbit thrombin to the small wounds.

It would appear desirable that these observations concerning the use of rabbit thrombin as a local hemostatic be extended.

**BLOOD: The Thrombic Activity of a Globulin Fraction Derived From Rabbit Plasma, Taylor, F. H. L., Lozner, E. L., and Adams, M. A. Am. J. Med. Sc. 202: 585, 1941.**

The observation of Parfentjev that rabbit plasma contains a substance or physiologic activity capable of converting fibrinogen to fibrin is confirmed.

Parfentjev's material is apparently a pseudoglobulin which possesses true thrombic activity.

It may be differentiated from thromboplastin by its manner of preparation and its independence of calcium-ion concentration in its action on fibrinogen.

Methods of preparing the material in a stable powdered form are presented.

**BLOOD CLOTTING: A Globulin Fraction in Rabbit's Plasma Possessing a Strong Clotting Property, Parfentjev, I. A. Am. J. Med. Sc. 202: 578, 1941.**

A description is given of a globulin fraction, isolated from rabbit plasma, which possesses the property of clotting plasma.

It has been found that the "toxicity" of this fraction on intravenous injection is due to intravascular clotting.

Clotting globulin could be separated from that fraction of rabbit plasma which contains the major portion of the immune bodies.

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## CLINICAL AND EXPERIMENTAL

### AGGLUTINATION REACTIONS IN RHEUMATOID ARTHRITIS\*

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BACTERIOLOGIC investigations in rheumatoid arthritis have been undertaken frequently since Billings, Rosenow, and others, advanced the theory of focal infection as a possible etiologic agent in this disease.

A considerable stimulus to this type of investigation was given by the work of Ceeil, Nicholls, and Stainsby,<sup>1</sup> who reported the isolation of a streptococcus from the blood stream of 61.5 per cent of 78 patients with rheumatoid arthritis—83.3 per cent of the positive cultures yielded organisms culturally and biologically identical—the “typical strain”—an attenuated hemolytic streptococcus.

Although others were unable to confirm this finding of positive blood cultures in rheumatoid arthritis, attention was turned to the fact that these “typical strains” of hemolytic streptococci were found by Nicholls and Stainsby<sup>2</sup> to be agglutinated in high dilutions by the sera of a large percentage of cases of this disease; 103 cases in 110 (93 per cent) had agglutinations of 1:640 or higher. Among 218 controls the majority were negative, and when agglutination was present it usually was in titer of less than 1:160. Hemolytic streptococci from other sources gave comparable reactions in lower titer. *Streptococcus viridans* gave weakly positive reactions. Sera from patients with other types of arthritis, febrile and nonfebrile diseases, and normal people, in most instances failed to agglutinate the organisms in significant titer.

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This work was repeated by Dawson, Olmstead, and Boots<sup>3-5</sup> who found no correlations between the source of the hemolytic streptococci and the agglutination titers obtained. Absorption tests failed to show specificity of the agglutination reaction for the strains of hemolytic streptococci examined. They found that in 206 patients with rheumatoid arthritis, 113 (54.8 per cent) had agglutinations of 1:160 or higher. A total of 256 control cases were negative or the agglutinations were of lower titer. They felt that there was a definite relation between the agglutination reaction and the age of the patient, and the duration of the disease. Examination of 41 other strains of gram-positive cocci failed to show comparable titers of agglutinations except tests using rough strains of pneumococci.

Blair and Hallman<sup>6</sup> examined the sera of 62 patients with rheumatoid arthritis and found that 82 per cent of them agglutinated "typical strain" AB66 in dilution of 1:160 or higher. Strains of hemolytic streptococci from other sources were agglutinated less frequently or in lower titers. A total of 32.9 per cent of 94 controls were positive in dilution of 1:160 or higher. They were unable to demonstrate any correlations between the agglutination titer and the age of the patient, the duration of the disease, the number of joints involved, or the sedimentation rate.

Gray and Gowen<sup>7</sup> found agglutination in some degree in 52 of 60 cases (86.6 per cent) against one of the "typical strains," AB13-45 (75 per cent) were in a dilution 1:160 or higher. The majority of cases of long standing showed agglutination in low titer, if at all.

Dawson and Wetherby<sup>8</sup> reported a strain of hemolytic streptococci isolated from a patient with chronic arthritis, agglutinated by 98.1 per cent of 60 persons with chronic arthritis and 28 per cent of controls in a titer of 1:400 or higher.

Cox and Hill<sup>9</sup> found that 197 sera from patients with rheumatoid arthritis agglutinated "typical strain" AB13 in approximately 70 per cent of cases in a titer of 1:320 or higher. Other organisms, including another strain of *Streptococcus hemolyticus*, *Streptococcus viridans*, *Streptococcus anhemolyticus*, *Staphylococcus aureus*, and *B. coli* were agglutinated in dilutions of 1:320 or higher in from 8 per cent to 49 per cent of cases.

McEwen, Bunim, and Alexander,<sup>10</sup> using a scarlet fever strain NY5, report positive agglutinations in 86 per cent of 36 patients with atrophic arthritis, 56 per cent in a dilution of 1:160 or higher. None of 35 normals had positive agglutinations.

Goldie and Griffiths<sup>11</sup> found that 80 per cent of 31 patients with rheumatic fever and chorea, 89 per cent of 28 patients with chronic infectious arthritis, 71 per cent of 17 patients with "streptococcal diseases," and 10 per cent of controls agglutinated stock strains of hemolytic streptococci in a dilution of 1:160 or higher.

Hartung, Davis, Steinbrocker, and Straub<sup>12</sup> report "typical strain" AB13 agglutinated by 36 per cent of 50 patients with rheumatoid arthritis 1:160 or higher. Two strains of hemolytic streptococci from other sources were agglutinated in 30 per cent and 24 per cent by the same sera. Ten per cent of normals were positive.



Tillett and Abernathy<sup>13</sup> found that in a number of bacterial infections other than streptococcal, the sera of the patients are capable of agglutinating certain strains of hemolytic streptococci. The reaction is demonstrable soon after the onset, persists during the active stage, and disappears after recovery. This agglutinability is striking when live organisms or organisms killed by formalin are used, but disappears if cultures are heated to the thermal death point.

Keefer, Myers, and Oppel,<sup>14</sup> therefore, performed a large group of agglutination tests using heat-killed organisms. The sera of 12 patients in 22 (55 per cent) with rheumatoid arthritis gave positive reactions; 9 patients (41 per cent) had agglutinations in a titer of 1:160 or higher. Only one serum agglutinated in a titer above 1:640. Positive reactions were obtained in 26.2 per cent of patients with rheumatic fever (42), 9.7 per cent of rheumatic heart disease,<sup>31</sup> 14.3 per cent of degenerative arthritis,<sup>28</sup> 23.5 per cent of erysipelas.<sup>17</sup> Sera from patients with other forms of arthritis, and a number of infectious and noninfectious diseases failed to agglutinate heat-killed streptococci. The reaction in patients with rheumatoid arthritis persisted for one to several months. In some cases it was maintained by intravenous vaccine.

Wainwright<sup>15</sup> performed a series of agglutinations with the sera of 51 patients with rheumatoid arthritis using both heat-killed and living hemolytic streptococci. He found the heat-killed organisms were agglutinated in slightly lower titers, but otherwise the curves were similar.

Wainwright,<sup>16</sup> in another investigation, found that agglutination of hemolytic streptococci by sera of patients with rheumatoid arthritis was not confined to organisms of group A (Laneefield), or to streptococci from human sources. Absorption with a representative strain of one group removed the agglutinins not only for that strain but for others of the same group—bearing out group specificity of the reaction rather than strain specificity.

McEwen, Chasis, and Alexander<sup>17</sup> did similar agglutination tests using strains of hemolytic streptococci of Laneefield's groups A, B, C, D, E, F, and G. The strongest agglutinations occurred in group A, but definite reactions were also obtained in the other groups. Crude C extracts of all groups were prepared, and when these were tested with the sera of patients with rheumatoid arthritis, occasional crossing was found with the extracts of all groups, and frequently with those of groups A, B, and G.

Chasis and McEwen<sup>18</sup> pursued this line of investigation further and found that cross reactions occurred with crude C extracts of hemolytic streptococci of the various groups, when used in precipitin tests with immune human sera and with immune rabbit sera, and the sera of patients with rheumatoid arthritis, rheumatic fever, and convalescent scarlet fever. This reaction was thought to be due to the presence of antibodies in the sera against a nongroup specific reaction present in the bacteria and in the crude C extracts, this substance being of nonprotein nature.

Dawson and Olmstead<sup>19</sup> examined the sera of 76 patients with rheumatoid arthritis for agglutinins against organisms in Laneefield groups A, B, C, D, E, F, and G. Using group A they found the percentage of positive reactions to be approximately the same as they had reported previously,<sup>4</sup> or about 55 per cent.

However, there were only three instances of definite positive reactions (1:160 or above) with organisms other than group A, and two of these were strongly positive for group A also. Control sera agglutinated groups B to G in as high dilution as did the sera of patients with rheumatoid arthritis. Control sera which agglutinated group A organisms in titers comparable with that of rheumatoid arthritis were found only in proved severe hemolytic streptococci infections.

Dawson, Olmstead, and Jost<sup>20</sup> compared the agglutinins and precipitins against hemolytic streptococci in 71 patients with rheumatoid arthritis and in 79 controls. They demonstrated a close approximation but not an absolute agreement between the capacity of rheumatoid arthritis sera to agglutinate strains of hemolytic streptococci and to precipitate various group-specific fractions of this organism. Bunim and McEwen<sup>21</sup> found no correlations between the anti-streptolysin titer and the hemolytic agglutination titer. Eighty-five per cent of 205 sera from 75 patients with atrophic arthritis had antistreptolysin titers within normal limits regardless of the stage of the disease or severity of clinical symptoms.

Short, Dienes, and Bauer<sup>22</sup> compared the results obtained in 49 patients with typical rheumatoid arthritis, using the sedimentation rate, Arneith-Schilling counts, the Vernes resorcinol test, and agglutination tests with hemolytic streptococci. A total of 92.2 per cent of the patients showed elevated sedimentation rates; 87 per cent of the Schilling counts were positive (over 8 per cent young cells), 58.3 per cent of the Vernes tests were positive; 53.1 per cent of agglutinations showed a titer above 1:160 for at least one of the four strains employed.

The evidence which has accumulated from these investigations shows that in rheumatoid arthritis there are, in a fairly large percentage of cases, positive agglutinations for hemolytic streptococci, regardless of the source of the organisms. This reaction comes on during the course of the disease, persists a variable length of time, varying in strength, and usually is low in titer or absent when the disease reaches a quiescent stage. Heat-killed streptococci are agglutinated similarly but in lower titer than with live organisms. Agglutinations are strongest with organisms of Lancefield's group A, but are present in other groups, and definite cross reactions frequently occur. There seems to be a close relationship between the agglutinins and precipitins for hemolytic streptococci, but none between agglutinins and antistreptolysins in rheumatoid arthritis. Elevated sedimentation rates and positive Schilling counts are considerably more constantly present than the resorcinol test and the agglutination reaction.

As indicated in the preceding reports, there is a wide variation in the results of the agglutination tests in different hands, and marked differences of opinion as to their significance.

Consequently, we performed a series of agglutination tests on patients with typical rheumatoid arthritis, with osteo-arthritis, with a number of related conditions, and on normal controls using a number of strains of hemolytic streptococci from various sources, and with two strains of *Streptococcus viridans*. This investigation was undertaken in an attempt to evaluate the agglutination reaction in relation to the patients seen in out-patient arthritis clinic.

## METHOD

The medium used for cultures was dextrose broth. Flasks of this media were inoculated with a diffuse fairly dense culture and incubated at 37° C. for fifteen to twenty-four hours.

Serum dilutions were made with 0.8 per cent sodium chloride solution in proportions of 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1,280, 1:2,560, and 1:5,120. To these dilutions 0.5 c.c. of culture was added, making the final dilutions 1:20, 1:40, 1:80, 1:160, 1:320; 1:640, 1:1,280, 1:2,560, and 1:5,120. A control consisting of 0.5 c.c. of culture and 0.5 c.c. of broth was included in all the tests.

TABLE I  
AGGLUTINATION TITER OF SERA OF PATIENTS WITH ARTHRITIS WITH HEMOLYTIC STREPTOCOCCI

| TYPE OF CASE                 | TITER OF AGGLUTINATION |      |      |      |       |       |       |         |         |         |             | PER-CENT-AGE POSITIVE | PER-CENT-AGE NEGATIVE |
|------------------------------|------------------------|------|------|------|-------|-------|-------|---------|---------|---------|-------------|-----------------------|-----------------------|
|                              | NEGATIVE               | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | 1:5,120 | TOTAL CASES |                       |                       |
| Typical rheumatoid arthritis | 3                      | 1    | 3    | 8    | 5     | 5     | 4     | 4       | 1       | 2       | 36          | 58                    | 42                    |
| Typical osteoarthritis       | 8                      | 1    | 4    | 5    | 4     | 2     | 0     | 0       | 1       | 0       | 25          | 28                    | 72                    |
| Related diseases             | 5                      | 0    | 1    | 1    | 1     | 0     | 1     | 0       | 1       | 0       | 10          | 30                    | 70                    |
| Controls                     | 19                     | 1    | 8    | 3    | 0     | 2     | 1     | 1       | 1       | 0       | 36          | 13.9                  | 86.1                  |

Agglutination tests were placed in a water bath at 56° C. for two hours and then overnight in the icebox. The tests were read at the end of two hours and after overnight refrigeration.

Readings were made after gently tapping the tubes until the bacteria had risen from the bottom. The reactions were recorded by the following scale:

- ++++ Disk or firm clump—broth clear.
- +++ Loose clumps, readily broken—broth clear.
- ++ Small clumps—broth clear.
- + Small but distinct clumps—broth cloudy.
- No clumps visible.

The highest dilution in which distinct and small clumps were observed was considered the end point or titer. The hemolytic activity of the strains employed was tested on blood agar.

Six strains of hemolytic streptococci were used in the agglutination tests. They were obtained from the following sources:

Strain I. From the throat of patient with scarlet fever.

Strain II. From patient with erysipelas.

Strain III. From throat of patient with rheumatoid arthritis and nephritis. This strain produces typical rheumatoid arthritis in rabbits.

Strain IV. From throat of patient with nephritis.

Strain V. From throat of patient with nephritis.

Strain VI. From dog tonsil.

Agglutination test was performed with at least three of these strains with the serum of each patient.

A smaller number of agglutination tests were performed with two strains of green streptococci obtained from the following sources:

Strain 1. From extracted tooth of patient with rheumatoid arthritis.

Strain 2. Blood culture from patient with subacute bacterial endocarditis.

The results are summarized in Table I. Agglutination titers of 1:160 or higher were obtained in 21, or 58 per cent, of 36 patients with typical rheumatoid arthritis. In 25 patients with typical osteo-arthritis, agglutination titers of 160 or higher were obtained in 7, or 28 per cent; in 10 patients with related diseases—rheumatic fever, fibrositis, and gonorrheal arthritis—positive agglutination reactions were obtained in 3, or 30 per cent. In the control group of 36 patients positive agglutination reactions were obtained in 5, or 13.9 per cent.

TABLE II

RELATIONSHIP BETWEEN DURATION OF DISEASE AND AGGLUTINATION REACTION IN RHEUMATOID ARTHRITIS

| DURATION OF DISEASE | NEGATIVE | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1280 | 1:2,560 | 1:5120 | TOTAL CASES |
|---------------------|----------|------|------|------|-------|-------|-------|--------|---------|--------|-------------|
| Six months or less  |          |      |      | 2    | 2     | 1     | 1     |        |         |        | 6           |
| One year            |          | 1    |      | 1    | 1     |       |       |        |         | 1      | 4           |
| One to five years   |          |      | 3    | 5    | 2     | 3     |       | 2      |         |        | 15          |
| Over five years     | 4        |      |      | 1    | 2     | 1     | 2     |        |         | 1      | 11          |
| Total               |          |      |      |      |       |       |       |        |         |        | 36          |

## DURATION

## One Year or Less

Positive—6 cases, or 60 per cent  
1:160 or higher

Negative—4 cases, or 40 per cent  
under 1:160

## Over One Year

Positive—13 cases, or 50 per cent

Negative—13 cases, or 50 per cent

TABLE III

AGGLUTINATION TITER OF SERA OF PATIENTS WITH ARTHRITIS WITH STREPTOCOCCUS VIRIDANS

| TYPE OF CASE                 | TITER OF AGGLUTINATION |      |      |      |       |       |       |         |         |         | PERCENTAGE POSITIVE |             |
|------------------------------|------------------------|------|------|------|-------|-------|-------|---------|---------|---------|---------------------|-------------|
|                              | NEGATIVE               | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | 1:5,120 |                     | TOTAL CASES |
| Typical rheumatoid arthritis | 9                      |      | 4    | 3    | 3     |       | 1     |         |         |         | 20                  | 20          |
| Typical osteo-arthritis      | 4                      |      | 3    | 1    | 1     | 1     |       |         |         |         | 10                  | 20          |
| Related diseases             | 7                      | 1    | 1    | 1    |       | 1     |       |         |         |         | 11                  | 9           |
| Controls                     |                        |      |      |      |       |       |       |         |         |         |                     |             |

The relation of the duration of the disease to the agglutination titer is shown in Table II. In this series there seemed to be no relationship between the two conditions. The results obtained with the two strains of *Streptococcus viridans* are summarized in Table III. Agglutination titers of 1:160 or higher were obtained in 4, or 20 per cent, of 20 patients with rheumatoid arthritis, with 2, or 20 per cent, of 10 patients with osteo-arthritis and in 1, or 9 per cent, of 11 controls.

## SUMMARY AND CONCLUSIONS

Previous experimental work on this subject is reviewed briefly. The agglutination titer of sera of 36 patients with rheumatoid arthritis, of 25 patients with osteo-arthritis, of 10 patients with related conditions, of 36 normal controls with strains of hemolytic streptococci obtained from various sources was determined. Agglutination titers of 1:160 or higher were obtained in 58 per cent of the patients with rheumatoid arthritis; in 28 per cent of those with osteo-arthritis, in 30 per cent of those with related conditions and in 13.9 per cent of the controls.

In this series there seemed to be no relationship between the duration of the disease and the agglutination titer.

Agglutination titers of 1:160 or higher were obtained with two strains of *Streptococcus viridans* in 20 per cent of 20 patients with rheumatoid arthritis, in 20 per cent of 10 patients with osteo-arthritis and in 9 per cent of 11 controls.

Although the percentage of positive agglutination reactions is considerably higher in patients with rheumatoid arthritis than in patients with osteo-arthritis, related conditions and controls, the reaction is of little value as a diagnostic measure, because patients with typical rheumatoid arthritis may have negative tests, and controls give positive reactions often enough to be confusing. The test is time-consuming, tedious, and difficult to perform accurately. No positive information is derived from agglutination reactions as to the etiology of the disease.

In the present state of immunological knowledge the significance of the agglutination reaction in rheumatoid arthritis is largely a matter of conjecture and is nothing more than suggestive. Whether infection by the hemolytic streptococcus plays a role of primary or secondary importance in the production of the disease, will require more evidence than that obtained from agglutination reactions.

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## INTRAMUSCULAR ADMINISTRATION OF SODIUM SULFAPYRIDINE TO CHILDREN\*

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**T**HOUGH a report<sup>1</sup> on the intramuscular administration of sodium sulfapyridine to patients appeared as early as July, 1939, this route has not been employed extensively. A recent favorable report<sup>2</sup> in this JOURNAL on the intramuscular route for parenteral administration of sodium sulfapyridine prompted the publication of our clinical experience and experimental work with the intramuscular administration of this drug.

Because such injections usually must be given by a physician, it seemed desirable to give them as infrequently as practicable. Our experience leads us to believe that in most infants and young children a satisfactory blood sulfapyridine level can be maintained by an intramuscular injection of sodium sulfapyridine no oftener than every twelve hours.

One hundred and ten intramuscular injections of a 30 per cent aqueous solution of sodium sulfapyridine monohydrate were given to 71 patients. The

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The sodium sulfapyridine was supplied by the Calco Chemical Company.

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dosages, calculated as sodium sulfapyridine monohydrate, were approximately as follows:

|   |
|---|
| 16 injections of 0.05 Gm. per kilogram of body weight |
| 75 injections of 0.1 Gm. per kilogram of body weight  |
| 18 injections of 0.2 Gm. per kilogram of body weight  |
| 1 injection of 0.3 Gm. per kilogram of body weight    |

When the total volume to be given amounted to more than 10 c.c., the dose was divided and given into two sites. Most of the injections were given as an initial dose to patients who were critically ill with pneumonia, and usually oral administration of sulfapyridine was begun within a few hours. The injections were given deep into the gluteal or quadriceps muscles. The number of injections in each patient was as follows:

|                                  |
|----------------------------------|
| 53 patients received 1 injection |
| 9 patients received 2 injections |
| 4 patients received 3 injections |
| 1 patient received 4 injections  |
| 2 patients received 5 injections |
| 1 patient received 6 injections  |
| 1 patient received 7 injections  |

For 21 patients oral administration of sulfapyridine was withheld for one to twenty-four hours in order to determine the course of the blood sulfapyridine level after a single intramuscular dose (Table I).

TABLE I

BLOOD SULFAPYRIDINE LEVELS FOLLOWING A SINGLE INTRAMUSCULAR DOSE OF A 30 PER CENT AQUEOUS SOLUTION OF SODIUM SULFAPYRIDINE MONOHYDRATE

The dosage is expressed as grams of sodium sulfapyridine monohydrate per kilogram of body weight.

| CASE | WEIGHT<br>KG. | DOSE<br>GM./KG. | FREE SULFAPYRIDINE LEVELS IN MG./100 C.C.<br>OF BLOOD |    |    |    |     |
|------|---------------|-----------------|---|----|----|----|-----|
|      |               |                 | HOURS AFTER INJECTION                                 |    |    |    |     |
|      |               |                 | 1   | 4  | 6  | 12 | 24  |
| 1    | 18            | 0.06            | 3   |    |    | 3  |     |
| 2    | 22            | 0.06            | 2   |    |    | 3  |     |
| 3    | 6.7           | 0.1             | 8   |    |    |    |     |
| 4    | 6.8           | 0.1             | 3   |    |    | 4  |     |
| 5    | 7.2           | 0.1             | 4   |    | 8  |    |     |
| 6    | 8.5           | 0.1             | 5   |    |    |    |     |
| 7    | 11            | 0.1             | 3   | 5  |    |    |     |
| 8    | 12            | 0.1             | 5   |    | 8  |    |     |
| 9    | 15            | 0.1             |   |    |    | 7  |     |
| 10   | 16            | 0.1             | 7   |    |    |    |     |
| 11   | 16            | 0.1             | 5   |    | 7  | 5  |     |
| 12   | 18            | 0.1             | 4   |    | 8  | 5  | 0.8 |
| 13   | 19            | 0.1             | 3   |    | 8  | 5  |     |
| 14   | 22            | 0.1             | 4   |    |    |    | 0.9 |
| 15   | 25            | 0.1             | 5   |    |    |    |     |
| 16   | 9.4           | 0.2             | 6   |    | 11 | 8  |     |
| 17   | 15            | 0.2             |   |    |    |    | 3   |
| 18   | 18            | 0.2             | 6   |    | 10 | 12 |     |
| 19   | 19            | 0.2             | 9   | 13 | 12 | 6  | 4   |
| 20   | 22            | 0.2             | 9   | 12 | 11 | 9  | 2   |
| 21   | 13            | 0.3             | 8   |    |    | 15 | 5   |

The local reaction in most cases was surprisingly slight. Considerable pain was a complaint during the injections, but it subsided within a few minutes. Slight tenderness and, in a few cases, some induration at the site of injection persisted for a day or two. Due to a misunderstanding one injection was given subcutaneously, resulting in a slough about 3 cm. in diameter. The patient had a staphylococcus bacteremia, which resulted in death a few days later. In one fat infant a deep slough about 3 cm. in diameter followed an injection of 0.2 Gm. per kilogram of body weight (6 c.c.) into the gluteal region. Since the depth of the subcutaneous fat in this infant was nearly as great as the length of the needle used, it is possible that the solution was not given into the muscle. In another infant the skin at the site of the needle puncture became necrotic for an area of about 1 cm. in diameter; this healed without ulceration.

If one uses the intramuscular route sloughs from accidental subcutaneous administration must be expected occasionally, but with proper precautions they should rarely occur. It is important to remember that on a well-nourished infant the subcutaneous fat in the gluteal region may be more than an inch thick.

From two patients who died one day after injection, specimens of muscle were obtained from the injected sites. The only gross changes noted were hemorrhage into the muscle in both cases and the deposition of a few small crystals in one. Microscopically there were hemorrhage, infiltration with polymorphonuclear neutrophils, and swelling and loss of cross striation of the muscle fibers.

In order to obtain further information regarding the severity of the damage to the muscle, each of ten rabbits was injected with 5 c.c. of the solution into the extensor muscles of the thigh. Although this volume amounted to a very large dosage for the rabbits, it was chosen because it represented about the average volume given to patients. The rabbits were autopsied at intervals of one to thirty-three days after the injections. The reaction in the muscle in all the rabbits was limited in the main to an area about 3 cm. in diameter. In some rabbits there were changes along the fascial planes for a slightly greater distance.

Rabbits autopsied on the first, second, third, and fifth days showed similar changes, except that crystals were found in the muscle only in the first rabbit. The crystals extended throughout an area about 1 cm. in diameter. Hemorrhage was the only other gross change noted. Microscopically, there were hemorrhage, edema, infiltration with polymorphonuclear neutrophils, and round cells, and loss of the staining properties of the muscle fibers.

The gross change in the muscles of the rabbits autopsied on the ninth, thirteenth, eighteenth, and twenty-third days consisted only of a brownish discoloration. Microscopically, there were degenerative changes in the muscle fibers; infiltration with polymorphonuclear neutrophils, round cells, and giant cells; and some increase in fibrous tissue.

The muscles from the two rabbits autopsied on the thirty-third day showed no gross changes, but microscopically, there was an infiltration with giant cells and an increase in fibrous tissue.



## CONCLUSION

A 30 per cent aqueous solution of sodium sulfapyridine monohydrate may be given intramuscularly to children when other routes of administration are not practicable. Following a dose of 0.1 Gm. per kilogram of body weight, a blood sulfapyridine level between 5 and 10 mg. per cent may be expected throughout most of the first twelve hours. The danger of a slough should not be great if proper precautions are taken to insure injection into the muscle. Although crystals (presumably sulfapyridine) may precipitate at the site of the injection, blood level studies on patients suggest that most of the solution is absorbed during the first twelve hours, and autopsy studies on rabbits demonstrate that crystals do not remain long at the site of the injection. The intramuscular injection of sodium sulfapyridine results in prolonged and perhaps permanent damage to the muscle, but the area involved is not large. The pathologic studies suggest that it may be undesirable to give more than one injection into any one site.

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## I. DAILY VARIATIONS IN THE TOXICITY OF NEOARSPHENAMINE IN RABBITS\*

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IN THE last decade Petersen<sup>1</sup> has shown that the "normal fluctuations" in human blood chemical levels are definitely interrelated and correlated with the weather. He also called attention to the possibility of variations in response of human beings to drugs in connection with this constantly changing physiologic state of the body and on his suggestion experimental investigations with morphine sulfate on mice were performed (Nedzel,<sup>2,4</sup> and Sargent and Nedzel<sup>3</sup>). It was found that the meteorologic conditions definitely varied the toxicity of morphine sulfate. Previously Maecht<sup>5</sup> found a very definite influence of barometric pressure and other meteorologic conditions on the potency of digitalis for cats, and pointed out that the difference in the toxicity is due to changes in the physiologic functions of the cats, more particularly to changes produced in the respiration and circulation by the fall in barometric pressure. He concluded that fluctuations in barometric pressure and other changes in atmospheric conditions play an important role in the action of various drugs.

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Here we present the observations on rabbits, which were administered neoarsphenamine. The animals used were of the same breeds, approximately of the same age. The females were all virgins, and in each daily group the same number of male and female animals was used. All animals were kept in an air-conditioned room (controlled temperature) under the same care. The diet consisted of Vitality Rabbit Ration in pellets, lettuce, and carrots. Water was supplied freely.

The experiments were performed in groups. Each day the same number of animals was injected with the same dose of neoarsphenamine per weight. The neoarsphenamine used in all these experiments was of the same lot, dissolved under similar conditions. The solution of the neoarsphenamine was never older than six minutes from the time the ampoule was opened, and was given intravenously in the ear vein. The injections were given at the same time in the morning.

The dose selected was established on the basis of an average death rate of approximately half of the number of the animals injected and was found to be 0.42 Gm. of neoarsphenamine per kilogram weight of the rabbit, as best to serve our purpose.

*Winter Group.*—One hundred and twenty rabbits were used in the first series, 10 rabbits being injected daily for twelve consecutive days, beginning February 17. The second series of experiments, which was started September 19, contained 150 rabbits, and the procedure was the same, namely, every consecutive day for fifteen days, 10 rabbits were receiving the same dose of neoarsphenamine as in the first group. The date of injections and the date of death, as well as the maximal and minimal daily temperature and barometric pressure for these days, are presented on Chart 1.

In the upper part of the chart are recorded the days on which the rabbits were given the neoarsphenamine intravenously, and are designated by a black dot. Each cross represents the death of one animal and is plotted on the day when this death occurred. The black lines, starting from the black dot, are extended to the right, and the crosses on these lines represent the death of a single animal on a certain day and indicate the group to which the animal belonged. Below this, the wide black field with white line in the center indicates the official temperature. The upper margin gives daily maximum temperature; the lower, the minimum temperature; the middle white line presents the mean temperature.

The lower curve indicates the official barometric pressure. Vertical lines relate days when many animals died with the corresponding temperature and barometric pressure. These lines are numbered to facilitate the discussion of events happening on the designated dates.

Arrow 1 connects the elevated peak of the barograph, low temperature, and points to the death of 4 of 10 injected rabbits. This is a day when cold air masses were passing and primarily a pressor phase existed in the animals. Line 2 shows death of 2 rabbits on the day of the injection of neoarsphenamine. At this time the meteorologic condition has been reversed in comparison with the previous day, the barometric pressure was low, and the temperature had reached a crest. Line 3 points to another 2 deaths on the same day of administration of drug; on this day the meteorologic conditions were quite different

from those of the previous day, namely, the temperature had fallen and the barometric pressure had increased rapidly. On line 4 four deaths occurred in three groups of animals previously injected. The lowest temperature and the highest barometric pressure for the period existed at this time.

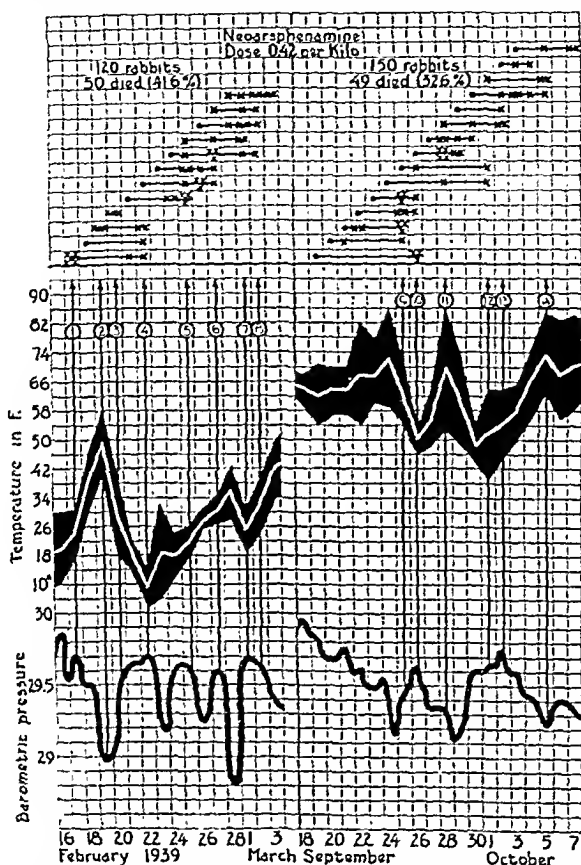


Chart 1.—Mortality protocol of rabbits injected with neoarsphenamine. Spring and autumn groups. Meteorogram below. Upturned arrows indicate days of increased mortality.

Line 5 points to 8 deaths that occurred in five groups of the previously injected animals. One death occurred in the animal injected on this day; at this time the crest of the polar air mass had just passed, and barometric pressure was falling. On line 6 there were 7 deaths in five groups, one of the deaths occurring on the day of injection. The temperature was slowly rising, but the barometric pressure reached its peak, sharply rising from the previous day. Line 7 points to 7 deaths in five groups. There was a sudden fall in temperature and a sharp rise in barometric pressure. From this day on there were no more daily injections. On March 8 there were 5 deaths in four groups.

*Autumn Group.*—In the autumn group the death rate was lower and, as can be seen in Chart 1, the temperature was on the whole much higher and the barometric pressure varied less. Yet during this period there were certain days on which the deaths varied in comparison with the others.

Line 9 shows 9 deaths in four daily groups; a polar front passed at this time, the temperature began to fall and the barometric pressure increased. On

the next day (line 10) 6 deaths occurred in four groups. A temperature minimum was reached and the barometric pressure crest occurred. Line 11 points to 8 deaths which occurred in four groups, one death occurring on the day of injection. Though the barometric pressure on this day declined slowly, the difference between the maximum and minimum temperatures was very great, indicating the disturbed atmospheric conditions at this time. Line 12 shows only 3 deaths occurred in three daily groups, but one death occurred on the day of injection. The temperature was increasing and the barometric pressure was still increasing. On line 13 there are shown 4 deaths in three groups, and a slowly rising mean temperature; barometric pressure reached its highest peak. Line 14 shows 4 deaths in three groups and a large difference between maximum and minimum temperatures, and the lowest barometric pressure in six days. No injections were made on this day.

The following conclusions may be drawn:

1. In late winter the neoarsphenamine is more toxic for rabbits in comparison with the same dose given under similar conditions in the autumn.
2. Toxic effects of neoarsphenamine are more marked on the days when the temperature is low and the barometric pressure is high (polar air mass).
3. Neoarsphenamine is also more toxic when the temperature is unusually high and the barometric pressure falls abruptly.
4. It is also somewhat more toxic when the difference between the maximum and minimum temperatures is great.
5. Presumably atmospheric interfaces are associated with greater susceptibility on the part of the animals. The aforesaid is true for deaths occurring immediately after the injection of neoarsphenamine as well as for delayed deaths.

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## II. DAILY VARIATIONS IN THE TOXICITY OF NEOARSPHENAMINE IN WHITE RATS\*

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IT HAS been shown that the toxicity of neoarsphenamine varies considerably by days in rabbits.<sup>1</sup> Herein are presented observations on white rats. The animals were of the same breeds, approximately of the same age. The females were virgins, and in each daily group the same number of male and female animals were used. The animals were kept in an air-conditioned room (controlled temperature) under the same care. The diet consisted of dog food cubes (given daily), lettuce—three times a week, and raw meat once a week. Water was given freely.

The experiments were performed in groups. Each day the same number of animals was injected with the same dose of neoarsphenamine, which was established on the basis of an average death rate of approximately half the number of the animals injected. The dose used was 0.225 Gm. per kilogram of weight and the injection was made at the tail. The neoarsphenamine used in all these experiments was of the same lot, dissolved under similar conditions. The solution of the neoarsphenamine was never older than six minutes from the time the ampoule was opened. The injections were administered at the same time in the morning.

The experiments on the white rats were performed in two series, the first one from May to June, and the second one from September to October, 1939. One hundred animals were used in the first series, 10 rats being injected daily for ten consecutive days, beginning May 24. The second series of experiments contained 140 animals, 10 animals per day, and began on September 19. The date of injections and the date of death, as well as the maximal and minimal daily temperature and barometric pressure for these days, are presented in Chart 1.

In the upper part of the chart are recorded the days, designated by black dots, on which the rats were given the neoarsphenamine at the tail. Each cross represents a death of one animal and is plotted on the day when this death occurred. The black lines, starting from the black dot, are extended to the right, and the crosses on these lines represent the death of a single animal on a certain day, and indicate the group to which the animal belonged.

*First Group.*—Line 1 (May 26) shows 4 deaths in two daily groups. At this date the barometric pressure was at its crest. Line 2 (May 29) reveals 4 deaths in three daily groups. Low temperature and high barometric pressure existed. Lines 3 and 4 (May 30 and 31) show 4 deaths in three daily groups for May 30, and 6 deaths in four groups for May 31. On these two days we had noticeable atmospheric disturbance indicated by great differences between the maximum and minimum temperatures. With line 5 (June 1) 9 deaths

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were observed in five groups. The temperature was falling and barometric pressure was rising (polar air mass). On line 6 (June 2) 11 deaths in six daily groups are noted. Difference between maximum and minimum temperatures was well pronounced, and the mean temperature decreased slowly. Line 7 (June 3) indicated 14 deaths in seven daily groups. There was a fall in temperature and a sharp rise in barometric pressure (polar air mass).

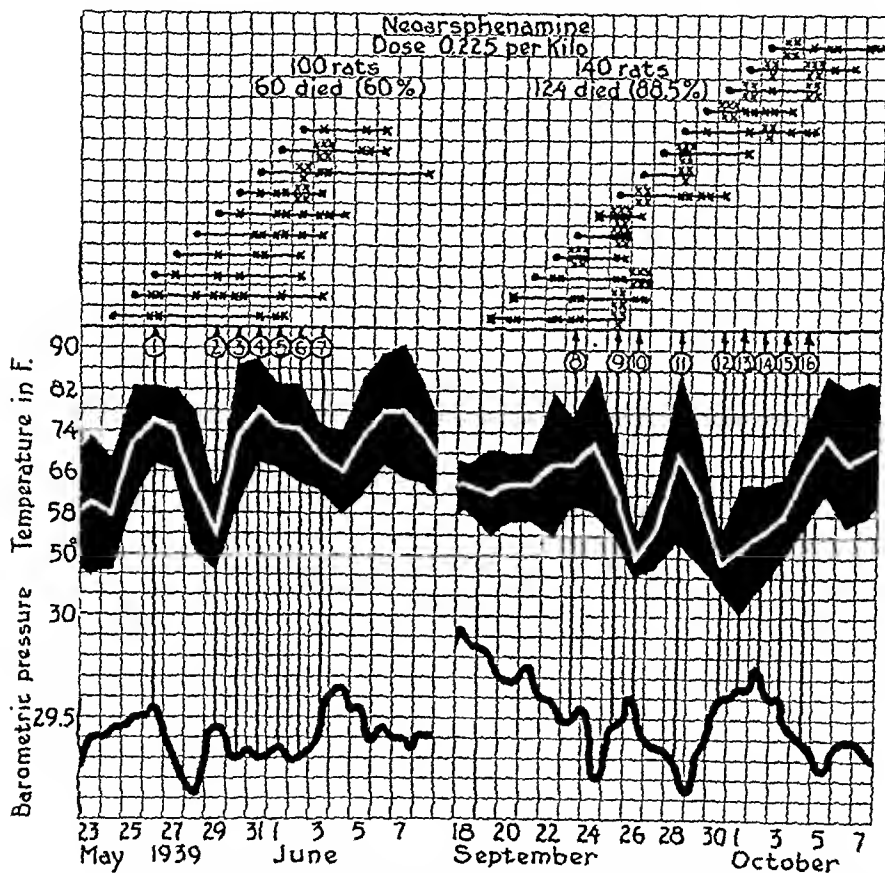


Chart 1.—Mortality protocol of rats infected with nearsphenamine. Spring and autumn groups. Upturned arrows indicate days of increased mortality.

*Second Group.*—In the September to October group line 8 (September 23) indicates 12 deaths in three daily groups. On this day there was a drop in temperature and the difference between maximum and minimum temperatures was pronounced. Lines 9 and 10 (September 25 and 26) reveal the passage of a major polar air mass. Twenty-seven deaths are indicated in six groups on September 25, and 13 deaths in four groups on September 26. Line 11 (September 28) reveals 12 deaths in three daily groups. The difference between maximum and minimum temperatures on this day was large. Line 12 (September 30) falls on the day of a polar front; 6 deaths were recorded in two daily groups. Lines 13 and 14 (October 1 and 2) indicate 8 deaths in four groups, and 9 deaths also in four groups. The barometric pressure was still rising, the temperature was comparatively low, and the difference between

maximum and minimum temperatures was pronounced. Lines 15 and 16 (October 3 and 4) show 6 deaths in three groups (October 3), and 12 deaths in four daily groups (October 6). On these days the temperature was rising and barometric pressure was falling. Disturbance is indicated by the large differences in maximum and minimum temperatures.

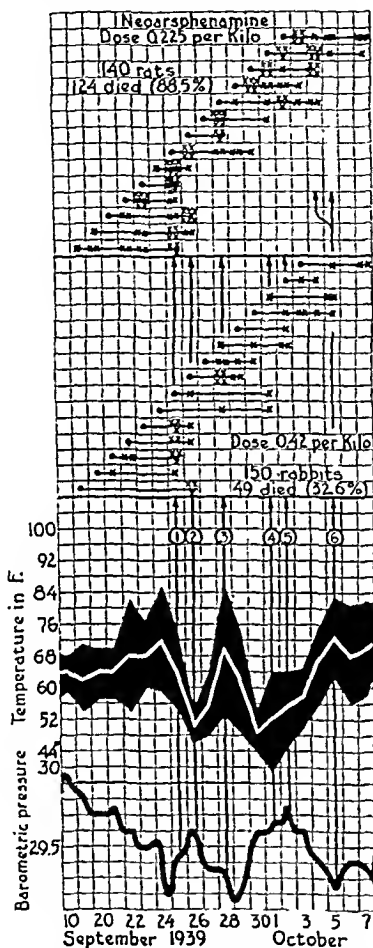


Chart 2.—Graph to illustrate similar trends in mortality for the autumn rat and rabbit groups.

From these observations the following conclusions may be drawn:

1. Neoarsphenamine is more toxic to white rats when meteorologic disturbances at the time of its administration are more pronounced.
2. The most toxic effects of neoarsphenamine in white rats are found to be on the days when the temperature is low and the barometric pressure is high (passing of polar air masses).
3. Neoarsphenamine is more toxic on the days when the temperature rises rapidly and the barometric pressure falls abruptly.
4. Neoarsphenamine is also somewhat more toxic when the difference between the maximum and minimum temperatures is great.
5. Increased toxicity holds for the deaths occurring immediately after the injection of neoarsphenamine as well as for delayed deaths.

*Rabbits and Rats of September-October Group.*—During the experimentation from September to October, 1939, it was possible to give the injections to rabbits (Nedzel) and white rats simultaneously. The results are presented in Chart 2. It becomes quite evident that the days when a larger number of deaths occurred among rabbits, a larger number of deaths among the rats also occurred (lines 1, 2, 3, 4, 5, and 6).

The following conclusions seem justified:

1. Similar environmental situations increase the toxicity of neoarsphenamine for different kinds of animals (in this case, rabbits and white rats).
2. Some days when rabbits survived or died in smaller numbers, were more fatal for the rats because of their greater susceptibility to the neoarsphenamine.
3. The days on which rats survived or died in a small number coincided with similar lessening of deaths for rabbits.

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## A RESPIRATION TAMBOUR WITH SOME UNIQUE FEATURES\*

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MANY varieties of respiration tambour are now in use in teaching and research laboratories. It is safe to say, however, that there is room for improvement in this type of apparatus. In teaching laboratories the simply constructed apparatus is preferred. Because it is handled by inexperienced hands, a breakdown of the apparatus occurs frequently in the middle of an experiment.

A respiration tambour should give satisfactory recordings from large animals, such as dogs, as well as small animals, such as rabbits. A correct recording from any kind of pneumograph or stethograph is advantageous. The cost of the apparatus should be reasonably cheap. The apparatus herein described meets these requirements. In addition it is easily adjusted. This eliminates confusion and may save the experiment.

This tambour is shown in Figs. 1, 2, and 3. The air cup *P* is cone-shaped, 42 mm. by 35 mm. This shape appears to eliminate some vibration of the writing lever. It produces a satisfactory record on the kymograph paper even with small animals, from which a good record is sometimes hard to obtain with many tambours.

An important adjustable feature is a flat piece, 35 mm. by 7 mm. by 4 mm.; *A*, Fig. 1, with one end fastened to the bottom of the tambour cup with an adjustable screw *B*. This enables arm *A* to be swung into any horizontal position. The opposite end of *A* is of different dimensions, 8 mm. by 7 mm. by 7 mm. Through a hole in this end, a vertical rod *C*, 80 mm. by 4 mm., is fitted.

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The bottom end of this rod is smaller in diameter than the top, the lower 30 mm. being 3 mm. in diameter instead of 4 mm., as it is at the top. This prevents the rod from slipping down in its entire length when being adjusted. A screw, *D*, in the end of the bottom piece, *A*, clamps the rod in the desired position. This rod adjusts the position of the writing lever in the horizontal plane. The top of the rod *C* is square; *E*, Fig. 2, measures 8 mm. by 7 mm. by 7 mm. Through a hole in this end is a horizontal rod *F* (Fig. 2), 70 mm. by 3 mm. One end of rod *F* is threaded and holds the lever assembly *G*. Movement of the lever assembly *G* about the axis of rod *C*, and by means of adjustment of the distance of this assembly from *E* accomplishes the change of position of the lever assembly to either side of the assembly *I*, which transmits the force from

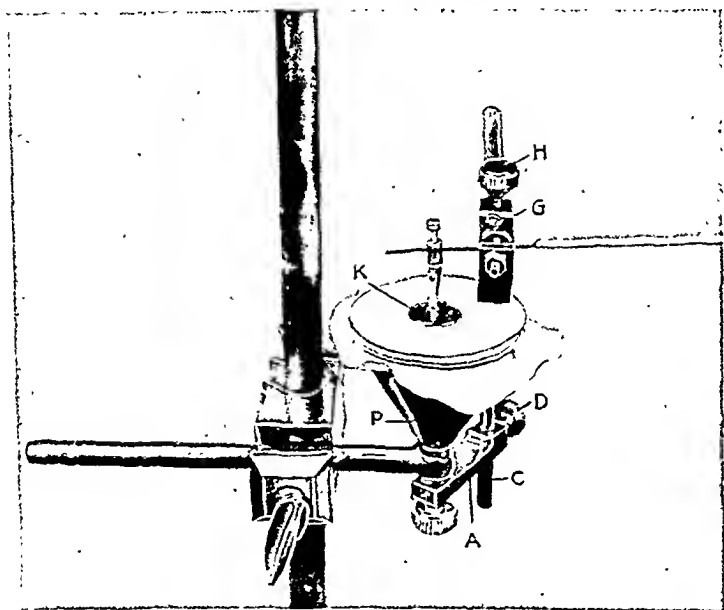


Fig. 1.—Lever joint is placed in front of the connection pin, reversing the direction of the writing level from the usual arrangement.

the tambour to the writing lever, *J*. Rod *F* is clamped in position with screw *H* on the end of vertical rod *C*. This change in position of the lever assembly *G* with reference to the fulcrum assembly *I*, enables one to change the direction of the movement of the lever. And in this way an adjustment to any type of pneumograph or stethograph can be made to obtain an upward movement of the record to represent inspiration. So far as we know this is a unique feature of this apparatus.

The footplate *K*, which is cemented to the rubber diaphragm, is connected to *I* by means of a swivel joint. This arrangement prevents the tearing loose of the footplate from the diaphragm when adjustments of the writing lever are made. This feature is also unique and can be used on any kind of tambour.

The writing lever *J* consists of an ordinary aluminum or straw heart lever. It is attached at *L* to the permanent portion of the lever assembly. It is, however, attached to the pivot *M* of the lever assembly with a small screw *N* set in a block *O*, in the same manner as an open-jawed support clamp. By this means,

the lever may be moved not only backwards and forwards but it may also be lifted out of the block *O* from any position. The block *O* which holds the lever is also attached to pivot *M* by the same arrangement.

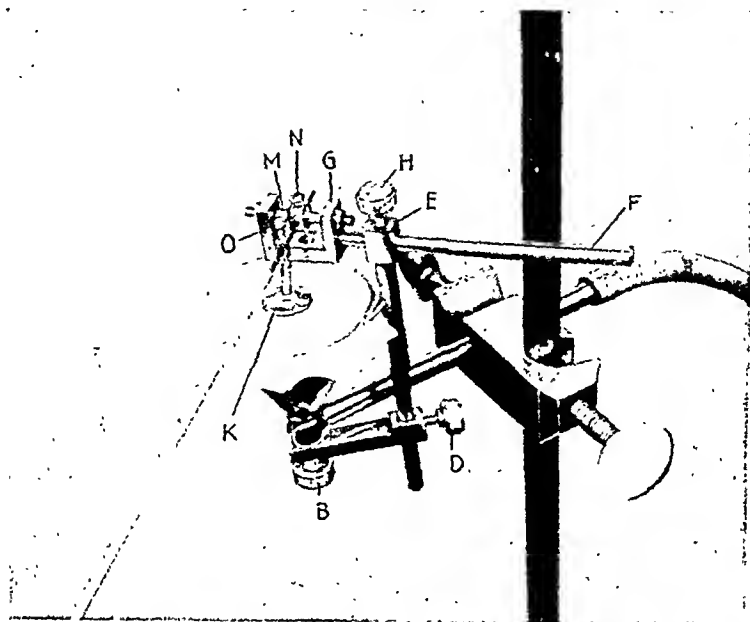


Fig. 2.—Writing lever turned in one of the many directions in which it can be placed.

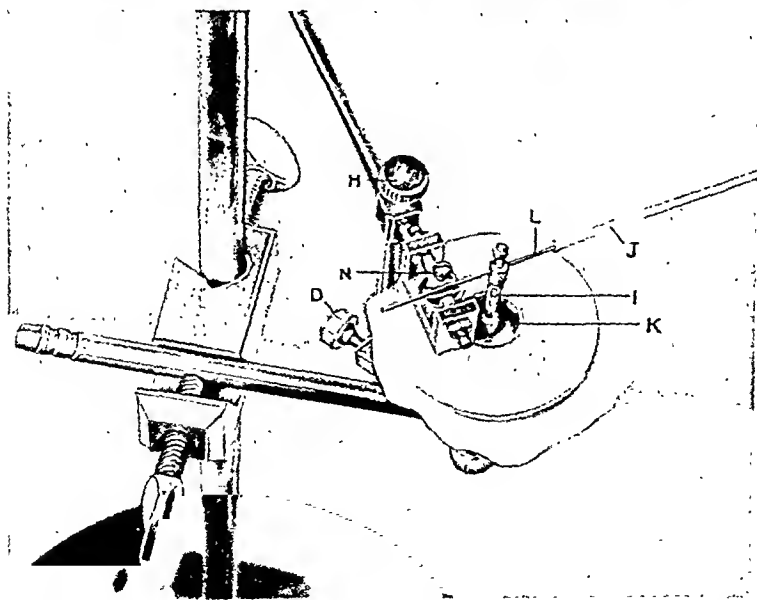


Fig. 3.—The lever joint is placed behind connection pin as in the usual tambour arrangement.

The apparatus is made of sheet copper and brass rod which are nickel-plated. The cost is reasonable and the apparatus is easily made by any competent mechanic.

## THE SEDIMENTATION RATE IN GOUT\*

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THE sedimentation rate of erythrocytes has been investigated thoroughly in most rheumatic states, but it has not been studied to any extent in gout. Hill,<sup>1</sup> and Gibson and Kersley,<sup>2</sup> report the sedimentation rate in a fairly large series of cases of gout. A few other reports, representing small series of cases, have appeared. Here, however, the records relative to the sedimentation rate in gout have been presented only as interesting observations incidental to the study of joint disease as a whole.

### PROCEDURE

We have studied the sedimentation rate in 100 consecutive cases of gout; 21 of these were patients with tophaceous gout and 79 were of the pretophaceous type. The diagnosis in the latter group was established by careful consideration of the history and physical and laboratory findings. The patients also were studied with reference to the activity of the disease at the time the examination was made. The level of uric acid in the blood was likewise determined.

For comparison, the sedimentation rate was measured in 50 apparently normal persons, in 100 consecutive cases of rheumatoid arthritis, and in 100 consecutive cases of osteoarthritis.

The erythrocyte sedimentation rate was determined by the method of Rourke and Ernstene<sup>3</sup> in which correction is made for the red blood cell volume. The upper limit of normal is considered 0.45 mm. per minute. The blood uric acid determination was made by the Morris-MacLeod<sup>4</sup> technique and 2.5 mg. per cent was taken as the upper limit of normal.

### RESULTS

The sedimentation rate in 100 cases of gout in all stages of activity ranged from 0.22 to 2.30 mm. per minute, with an average rate of 0.94 mm. per minute; 87 per cent of the patients showed an increase in the rate above the upper limit of normal of 0.45 mm. per minute (Table I).

The average sedimentation rate found in the normal group, in the group with osteo-arthritis, and in the rheumatoid group was 0.24, 0.51, and 0.98 mm. per minute, respectively. These are shown and compared with the gout group in Table II.

In Table III is recorded the average sedimentation rate found when the cases of gout were classified according to the activity of the disease. The average

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blood uric acid for each of these groups is noted also. The average rates found are 1.13, 0.87, 1.21, 1.02, and 0.68 mm. per minute for the acute, acute subsiding, chronic progressive, chronic, and inactive groups, respectively. The average blood uric acid values are 3.8, 3.7, 3.3, 3.9, and 4.0 mg. per cent for each group given in the same order.

TABLE I  
SEDIMENTATION RATE IN GOUT

| Sedimentation rate in millimeters per minute | Under 0.46          | 0.46-0.60 | 0.60-0.80 | 0.80-1.00 | 1.0 Plus |
|--|---------------------|-----------|-----------|-----------|----------|
| Number of cases                              | 13                  | 8         | 16        | 15        | 48       |
| Per cent                                     | 13.0                | 8.0       | 16.0      | 15.0      | 48.0     |
| Average sedimentation rate                   | 0.94 mm. per minute |           |           |           |          |
| Highest                                      | 2.30 mm. per minute |           |           |           |          |
| Lowest                                       | 0.22 mm. per minute |           |           |           |          |

TABLE II  
COMPARISON OF SEDIMENTATION RATES IN NORMAL PERSONS, ARTHRITIS, AND GOUT

| DIAGNOSIS            | NUMBER OF CASES | AVERAGE SEDIMENTATION RATE |
|----------------------|-----------------|----------------------------|
| Normal persons       | 50              | 0.24                       |
| Osteo-arthritis      | 100             | 0.51                       |
| Rheumatoid arthritis | 100             | 0.98                       |
| Gout                 | 100             | 0.94                       |

TABLE III  
ACTIVITY OF DISEASE, AVERAGE SEDIMENTATION RATE, AND AVERAGE BLOOD URIC ACID IN GOUT

|  | ACUTE | ACUTE SUBSIDING | CHRONIC PROGRESSIVE | CHRONIC | INACTIVE |
|--|-------|-----------------|---------------------|---------|----------|
| Number of cases                                      | 45    | 26              | 2                   | 9       | 18       |
| Per cent   | 45.0  | 26.0            | 2.0                 | 9.0     | 18.0     |
| Average sedimentation rate in millimeters per minute | 1.13  | 0.87            | 1.21                | 1.02    | 0.68     |
| Average blood uric acid in milligrams per cent       | 3.8   | 3.7             | 3.3                 | 3.9     | 4.0      |

#### DISCUSSION

Since the original investigations of Fahraens<sup>5</sup> on the sedimentation rate of erythrocytes, much interest has been shown in this property of the red blood cells. Many studies in rheumatic disorders have shown the sedimentation rate to be consistently elevated in rheumatoid arthritis, with only slight elevation in the osteo-arthritic type, probably not out of proportion to the advanced age of the persons in whom it occurs. The findings of others have been essentially in agreement with the average values cited in Table II, taken for comparison with our average values for gout.

In the occasional case of gout studied with reference to the sedimentation rate, there has usually been a tendency to an increased velocity of settling of the erythrocytes, but the striking uniformity in this increase in the rate and the magnitude of this increase probably has not been properly appreciated. Kahlmeter,<sup>6</sup> in a discussion on the sedimentation rate in rheumatic disorders,

reported observations with respect to this in 6 cases of gout, 3 active and 3 chronic. He found the sedimentation rates definitely elevated in the active cases with rates only slightly above normal in the chronic cases. He does not indicate clearly whether the cases in the latter group represent chronically active disease or whether they are merely cases of long duration with intermittent clinical activity. Hench<sup>7</sup> states that the sedimentation rate is elevated in gout but he does not further elaborate on this statement of fact. Hill,<sup>1</sup> reporting his observations on 93 cases, observed that the sedimentation rate varies considerably in gout, being markedly increased only during acute attacks and returning quickly to normal following the acute episode. Gibson and Kersley<sup>2</sup> found the erythrocyte sedimentation rate to be increased in 59 per cent of 68 cases of gout they studied. It is their belief that the sedimentation rate measures the factor of severity of the local tissue reaction.

Although the average rate found here for the 100 consecutive cases was 0.94 mm. per minute and an elevation above normal occurred in 87 per cent of the patients, it should be emphasized that the sedimentation rate may be found to be within normal limits in certain instances. Thirteen of the 100 patients had rates of 0.45 mm. per minute or less. However, in each instance the gout was either subsiding after an acute exacerbation or was classified as inactive. On the other hand, many cases of inactive and subsiding acute gout were seen in which the readings were decidedly elevated.

The sedimentation rate tends to reflect in part, at least, the activity of the disease. This interesting observation is drawn from examination of Table III, where it is seen that the highest average values in the common type of intermittently active gout occurs in the group representing the acute phase of the disease. As the process becomes less active, the average rate is reduced, as noted in the average values found in the acute subsiding and inactive groups. When the disease tends to be of a progressive nature, the sedimentation rate reaches its highest level. The observations made in this particular group, however, represent only 2 cases and are, therefore, subject to criticism. The average value found in the chronic group is about as would be expected, being neither extremely high nor as low as in the inactive group. The shift in the sedimentation rate noted with respect to the activity of the disease is, of course, not peculiar to gout. Essentially the same reaction is encountered in rheumatoid arthritis.

There is no adequate explanation for the elevation of the sedimentation rate in gout at the present time. Infection is certainly not a factor, for careful survey of these individuals for chronic foci has usually proved unsuccessful. Treatment or removal of infected teeth, tonsils, sinuses, or prostate glands had been carried out in 45 per cent of these cases before they came under our observation, without affecting the course of the disease. Gilligan and Ernestene<sup>8</sup> have demonstrated that the plasma fibrinogen level is the major factor in controlling the sedimentation rate. Assuming an increase in the plasma fibrinogen in gout, one may offer a possible explanation on a basis of faulty intermediary protein metabolism or perhaps on the basis of impaired liver function. A functional disturbance in the liver in gout does not seem unlikely, since these patients

are almost uniformly obese and are notorious for their indiscretion regarding diet and the use of alcohol.

An examination of the data, as shown in Table III, fails to reveal any parallelism between the sedimentation rate and the level of the blood uric acid which reflects in part, at least, the extent of metabolic dysfunction.

#### SUMMARY

1. The sedimentation rate was found to be elevated above 0.45 mm. per minute in 87 per cent of 100 cases of gout. The average rate for the series was 0.94 mm. per minute. The upper limit of normal by our method is 0.45 mm. per minute.

2. For comparison the sedimentation rate in 50 apparently normal persons, 100 cases of osteo-arthritis, and 100 cases of rheumatoid arthritis was determined. The average rates found were 0.24, 0.58, and 0.98 mm. per minute, respectively.

3. The sedimentation rate varies with the activity of the disease, being lower in acute subsiding cases than in acute cases, and reaching its lowest level in inactive cases. It reaches its highest level in the chronic progressive type of gout and an intermediate level in the simple chronic type of the disease.

4. There is no apparent correlation between the sedimentation rate and the degree of metabolic dysfunction, as indicated by the level of the blood uric acid.

5. The sedimentation rate may at times be of value in the differential diagnosis of gout, osteo-arthritis, traumatic arthritis, bursitis, tendonitis, fibrositis, and peri-arthritis.

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# THE ROLE OF AUTO-ANTIBODIES IN THE SERODIAGNOSIS OF SYPHILIS\*

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THE history of the serodiagnosis of syphilis and the speculation as to the nature of the constituent of serum that reacts with tissue lipids in the present-day serodiagnostic tests for syphilis have been summarized by Eagle.<sup>9, 10</sup> From the 1937 summary<sup>9</sup> it seems controversial as to whether reagin is an antibody to lipid haptens of the host, liberated at foci of infection and activated by spirochetal protein, or an antibody to the spirochete which contains a lipid related to those found in mammalian tissues (and in vegetables,<sup>36</sup> as well as possibly in other bacteria and protozoa, the so-called ubiquitous lipid). Recent work with the spirochetal complement-fixation test<sup>10</sup> seems to indicate that the spirochete of syphilis stimulates the production of at least two *separate* antibodies, (a) antilipid and (b) antispirochetal, both in response to substances contained in the spirochete.

With no thought of disparaging this admirable work, but rather with the hope of emphasizing its potential significance, exception must be noted to the implication of the repeated statement that "there seems no a priori reason why spirochetal protein should uniquely activate the lipoidal tissue hapten and thus initiate antibody production, while in other conditions in which there is just as much tissue destruction and in which some other bacterial protein is available as the activator, the Wassermann remains consistently negative."<sup>9, 10</sup> While there is indeed no apparent "a priori reason," the implication that serodiagnostic tests for syphilis remain consistently negative in other conditions is not beyond question.† From the reports of biologically positive reactions in certain nonsyphilitic diseases in man<sup>1, 6, 7, 9, 25, 26, 28, 32-34</sup> and lower animals,<sup>24</sup> the occurrence of positive reactions in apparently normal lower animals<sup>9, 13, 31</sup> and the possible occurrence of reagin in normal human serum<sup>2</sup> all constitute evidence that must not yet be disregarded.

Rosebury<sup>12</sup> pointed out that, theoretically, spirochetal protein is not necessary for the activation of autogenous lipoidal haptens, and Kahn<sup>18</sup> emphasized the possibility that in the presence of tissue destruction lipid protein combinations may be liberated similar to those liberated in syphilis. He further pointed out that in rare instances traces of tissue proteins and lipids liberated in the process of metabolism might be sufficient to stimulate antibody production against lipids.

The production of auto-antigens within the living body of an experimental animal (guinea pig) by application of the physical agents of heat or cold has recently been reported by Karady<sup>19</sup> but has not been confirmed.

Reference has already been made to the occurrence of positive reactions in man in certain diseases other than syphilis. Particular attention is called to the report of Taoka<sup>33</sup> that positive serologic reactions were found in 24 per cent of 100 patients with carcinoma of the cervix uteri as compared with only 10.38 per cent of 106 general in-patients; also Stojalowski<sup>32</sup> found that of 830 cases studied by post-mortem examination there were 15 with no post-mortem sign of syphilis, which had been diagnosed and treated for syphilis. In 3 cases serologic tests were not done; in 3 cases serologic reactions were negative; and, in 7 cases serologic tests were done only in the moribund stage. In correlating the post-

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†Since this was written the following papers have been published:

Kahn, R. L.: A Serologic Verification Test in the Diagnosis of Latent Syphilis, *Arch. Dermat. & Syph.* 41: 817, 1910.

Moore, J. E., Eagle, H., and Mohr, C. T.: Biologic False Positive Serologic Tests for Syphilis, *J. A. M. A.* 115: 1602, 1910.

mortem histologic diagnoses with the serologic findings he found that: of 2 cases of carcinoma 2 were positive; of 5 cases of tuberculosis 2 were positive; and of 5 cases of myocarditis 2 were positive.

It has been reported that positive serologic reactions for syphilis have been induced in rabbits by the injection of various preparations. These include: killed cultures of *T. pallidum*;<sup>20, 21</sup> aqueous extract of syphilitic liver;<sup>5, 11, 16</sup> alcoholic extract of syphilitic liver<sup>11</sup> and syphilitic rabbit's testicle;<sup>13</sup> mixtures of lipid extracts from various normal human, rabbit, or beef organs with some foreign protein;<sup>4, 8, 15, 16, 30, 35</sup> a mixture of lecithin and hog serum;<sup>27</sup> washed floccules from positive reactions of human serum from syphilitic patients<sup>3, 8, 29</sup> and from malarial patients,<sup>29</sup> and of pooled bovine serum,<sup>29</sup> and syphilitic rabbit serum;<sup>3</sup> aolan;<sup>27</sup> and emulsions of hamster tissues.<sup>28</sup> Positive reactions in rabbits have also been noted as a result of experimental infection with bovine tubercle bacillus,<sup>24</sup> and have been induced in human beings by the injection of killed cultures of *T. pallidum*,<sup>22</sup> and killed trypanosomes.<sup>23, 38</sup>

Reports of unsuccessful attempts to produce artificial positive reactions in rabbits include the injection of alcoholic extracts of normal human heart and normal infant liver;<sup>11</sup> alcoholic extracts of (syphilitic ?) liver;<sup>16</sup> alcoholic extracts of normal rabbit's testicle;<sup>13</sup> turpentine, dilute alcohol, and *b*-tetrahydronaphthylamine;<sup>24</sup> and floccules from positive tests on normal rabbit serum,<sup>8</sup> and human leprous serum (one trial only).<sup>29</sup>

Because of the incidence of positive reactions in rabbits,<sup>9, 13, 24, 31</sup> it is difficult to interpret all of these reports. However, they seem to indicate that it is possible artificially to induce positive serologic reactions in rabbits by various methods, many of which in no way involve either *T. pallidum* or any specific derivative of it.

With these things in mind it appeared that the auto-antibody theory might account for positive reactions with lipid antigen in the absence of syphilis, and that it might be susceptible to investigation by animal experimentation.

#### EXPERIMENTAL

*Materials and Methods.*—Rabbits, after preliminary serologic study, were variously treated in attempts (a) to produce pathologic changes characterized by chronic inflammation and degenerative changes simulating the essential lesions of syphilis while observations of the reagin titer were made at frequent intervals; or (b) to stimulate or to augment the production of reagin by the injection of various extracts of homologous tissue or preparations of autogenous serum.

Necropsy has been performed upon these animals to observe any correlation between the serologic findings and the pathologic changes.

The serologic examination included the Kahn Standard, Kline Diagnostic, and the Mazzini flocculation tests; quantitative tests were done whenever a four-plus reaction was encountered. Complement-fixation tests were attempted, but the high incidence of anticomplementary reactions rendered the results almost worthless. For a time the sera were heated for thirty minutes at 62° C. instead of 56° C., in an attempt to reduce the incidence of these anticomplementary reactions. It was observed that heating at this higher temperature reduced not only the anticomplementary activity but also the reagin titer; consequently, this deviation from the regular technique was soon abandoned.



*Incidence of Serologic Reactions in Untreated Rabbits.*—The sera from 18 rabbits were tested after heating at 56° C. for thirty minutes; 2 were Kahn negative, 2 were Kline negative, and 6 were Mazzini negative. The reactions of the others ranged from a one-plus reaction to a titer of 5. Three rabbits were not tested with the complement-fixation test and 2 of them were anticomplementary in all reacting dilutions; of the 13 on which readings were obtained, 7 were negative.

The sera of 10 rabbits were tested after heating at 62° C. for thirty minutes; 10 were complement-fixation-negative, 8 were Kahn negative, 8 were Kline negative, and 8 were Mazzini negative. The same 2 rabbits reacted in the Kline and Mazzini tests; the 2 rabbits which reacted in the Kahn test were negative to all other tests.

From these groups animals were chosen for further study.

*Variation of Serologic Titer in Untreated Rabbits.*—Of the 8 rabbits first chosen for study all but one showed considerable variation of reagin titer, as demonstrated by the flocculation tests; one animal gave consistently negative results with all tests. A study of the records of these animals reveals that (a) upon repeated examination the various techniques did not show similar variation of titer, one rabbit excepted; (b) upon repeated examination with a given technique the various animals did not show a uniform variation from the titer of the previous examination with the same technique; and (c) in general, the Mazzini technique in repeated examinations of a given animal seemed to show a lower titer and less variation in titer than did either the Kline or Kahn test; one rabbit represents an exception to this observation and exhibited a lesion (echinococcal cyst) not found in the other animals.

While the possibility of some daily variation in the sensitivity of the various antigen emulsions cannot be completely ruled out, these observations are considered as sufficient evidence for the belief that this factor does not account for the variations in titer observed under treatment.

#### PATHOGENIC AND IMMUNOLOGIC PROCEDURES

1. *The injection of flocculate from positive human serum.* In order to secure a preliminary observation as a basis of comparison, a female rabbit, which had shown clearly negative results over a period of six weeks, was given weekly intravenous injections of washed flocculate from mixtures of Mazzini-antigen suspension and positive human serum. A total of 9 injections of floccules from 0.6 ml. of four-plus serum was given.

One week following the third injection the Kahn test showed a one-plus titer; the following week this had increased to a two-plus. Just before the fifth injection the Kline, Mazzini, and complement-fixation tests showed partial positives for the first time. The Mazzini test gradually rose to a titer of 10 before the eighth injection. During the following week it dropped sharply to one-plus.

2. *Intra-abdominal transplant of rabbit's aorta.* A freshly removed rabbit aorta was placed in the peritoneal cavity of a female rabbit. No abnormalities were observed, and clinical recovery was uneventful. The Kahn titer rose from

one-plus to two-plus two weeks after the operation, and was maintained at this level until the seventh week when it dropped again to one-plus. The Kline titer was slightly diminished during the first four weeks, returned to one-plus in the fifth week, rose slightly in the sixth week, and returned to one-plus in the seventh week. The Mazzini titer was slightly elevated in the fourth, fifth, and sixth weeks, and again dropped to negative in the seventh week. Necropsy in the eighth week showed an encysted granulomatous mass enclosing the transplanted aorta.

3. *Ligation of the splenic pedicle.* A female rabbit was subjected to laparotomy, at which time the splenic pedicle was ligated. No abnormalities were observed, and clinical recovery was uneventful.

The Kahn titer rose to three-plus in ten days and to four-plus in thirty-one days after the ligation. During the next week it dropped to one-plus. The Kline and Mazzini tests showed no significant variation of titer. The complement-fixation test was one-plus two and one-half weeks after surgery, but later examinations were again negative. Necropsy in the seventh week revealed replacement of the greater portion of the spleen by a nonspecific granulomatous mass exhibiting central necrosis resembling caseation.

4. *Injection of homologous tissue preparations.* The heart, aorta, liver, brain, and psoas major muscles of an untreated female rabbit were dehydrated in repeated changes of acetone. The dehydrated tissue was finely chopped and mixed for the preparation of the various extracts.

#### A. *Aqueous extract of homologous tissues*

Three grams of this dehydrated rabbit tissue were mixed with 50 ml. of 0.85 per cent sodium chloride solution and shaken mechanically for an hour. This mixture was filtered, the residue was discarded, and merthiolate, 1:40,000, was added. Two rabbits were given weekly intra-abdominal injections of this aqueous extract, starting with a dose of 1 ml. and increasing to 3 ml. in the seventh week. One rabbit showed no significant variation in serologic titer during eight weeks, nor were significant changes found at necropsy. The other rabbit showed some fluctuation in the Mazzini titer, and a gradual increase in the Kahn titer from negative at the end of the second week to three-plus at the end of the eighth week. This latter rabbit at necropsy exhibited a splenic lesion which consisted of a cystlike yellow mass containing dirty dark-brown cheesy material. Microscopically, it could be seen that the yellow color was due to yellow droplets in phagocytic cells which composed the inner portion of the "cyst" wall. These cells appeared to be large mononuclear cells, which in some areas exhibited a loss of cell outline and resembled syncytium. Multinuclear cells of the foreign-body type were seen. The etiology of this lesion remains obscure.

#### B. *Lipid extract of homologous tissues*

Fifteen grams of the dehydrated rabbit tissues were treated to obtain ether-soluble acetone-insoluble and ether-insoluble alcohol-soluble fractions which were mixed in alcoholic solution and portions used for injection after being quickly added to an excess of 0.85 per cent sodium chloride solution.

Two rabbits got weekly intra-abdominal injections of this extract, 0.5 ml. in each of the first four doses and 1.0 ml. in each of the last three. One of these rabbits after negative preliminary tests exhibited a gradual rise to a two-plus Kahn and a one-plus Kline and Mazzini; no significant lesions were found at necropsy. The other rabbit exhibited preliminary reactions varying between three-plus and a titer of 10. Under treatment the Mazzini titer was constant at four-plus; the Kline fluctuated from a level of three-plus; and the Kahn was at first diminished to a two-plus and then rose to a four-plus level. At necropsy an echinococcal cyst was found.

#### C. *Protein derivatives of rabbit tissues (peptic metaprotein)*

The residue of the rabbit tissues after ether and alcohol extraction as already described was treated with 150 ml. of 0.3 per cent hydrochloric acid and approximately 0.25 Gm. of pepsin. After ten minutes at 37° C. a small portion was separated, while the remainder was permitted to stand for four hours at this temperature. The material was filtered, neutralized with sodium hydroxide, and heated to boiling. The products of the ten-minute and the four-hour incubations were mixed. This preparation yielded positive biuret and xanthoprotein reactions; Millon's reaction was negative. A precipitate formed upon the addition of ammonium sulfate, but boiling did not cause precipitation. Merthiolate, 1:40,000, was added.

Two rabbits were treated with weekly intra-abdominal injections of this preparation, the doses increasing from 1.0 to 3.0 ml. in the ninth week. Both animals showed slight increases of the Kahn titer without significant change in the results of the other tests. Neither animal showed significant lesions at necropsy.

#### D. *Recombined lipid and peptic metaprotein from rabbit tissues*

A portion of the peptic metaprotein extract was treated by adding to it, drop by drop, during vigorous agitation, the lipid extract (B) until a preparation resembling clear yellow blood serum was obtained.

Six rabbits were treated with weekly injections of this preparation in doses increasing from 1.0 to 3.0 ml. in the ninth week. Two showed very slight transient increases of titer. Necropsy revealed in one cysticercoid cysts of the omentum and in the other a 5 mm. encysted granulomatous abscess located retroperitoneally. One showed fluctuation of the previously constant Kahn titer in an elevated range; necropsy revealed no significant lesions. Two showed primary diminution and secondary elevation of titer, most marked in the Kahn, but also evident in the other flocculation tests; necropsy revealed no significant lesions in one of these, while in the other occasional small areas of patchy necrosis were present in the renal cortex. One, after a previously negative reaction, showed a definite rise of titer, evident in all the tests and comparable to that seen in the rabbit injected with flocculate from human syphilitic serum. Aside from blindness, probably of glaucomatous origin, this rabbit exhibited no significant lesions.

5. *Injection of heated autogenous serum.* Two rabbits were treated with autogenous serum which had been heated one hour at 56° C. The dosage was

0.5, 0.75, 1.0, 1.0, 1.0, and 1.0 ml. at weekly intervals. In one rabbit these doses were reinforced with 0.2 ml. of a 5 per cent suspension of collodion particles prepared by the method of Zozaya.<sup>37</sup> Both animals showed a gradually increasing Kahn titer during the first four weeks, after which the titer dropped in each case. The Kline and Mazzini titer gradually decreased during the period of treatment. Neither rabbit exhibited significant pathologic changes at necropsy.

#### DISCUSSION

The production of a positive serologic reaction following the injection of floeculate from human syphilitic serum has been frequently reported and was readily confirmed. The fall of titer that occurred during the eighth week while the injections were still in progress does not seem to have been previously reported. Neither has the apparent partial specificity of the antibody produced for the particular antigen floeculate been emphasized.

The pathogenic procedures of aortic transplantation and splenic ligation were each successful in producing local lesions of the desired type. A somewhat similar lesion occurred, apparently spontaneously, in one of the rabbits treated with aqueous extract of rabbit tissues and may account for the changes in titer observed in this animal.

It is probably apparent that among the various extracts of rabbit tissues the lipid-metaprotein preparation constituted the real test antigen, while the other preparations were employed as controls. In connection with the apparent slight antigenic action of the lipid extract it should be mentioned that its freedom from proteins is probably questionable. The colloidal state in which the lipid was injected might possibly tend to increase its antigenicity.

While with the exception of one rabbit treated with lipid-metaprotein preparation these changes are not of convincing magnitude, they are all compatible with the auto-antibody theory and are best explained by it. Furthermore, the changes in human subjects where the auto-antibody mechanism may be the responsible factor are usually of similarly slight degree.

The significance of the spirochetal complement-fixation test, especially when done after preliminary absorption with lipids, becomes even greater in the face of such an auto-antibody theory.

#### SUMMARY

Attempting to demonstrate evidence which would support or controvert the auto-antibody theory in accounting for nonspecific reactions with lipid antigens in serodiagnostic tests for syphilis, rabbits were subjected to various treatment intended to stimulate or to augment the production of reagin by non-specific and noninfectious means.

1. Granulomatous lesions were produced in 2 rabbits by surgical procedures; both animals exhibited slight elevations of Kahn titer between the second and seventh weeks.

2. Of 6 rabbits treated with a lipid-metaprotein preparation, 2 showed very slight transient increases of titer, one showed fluctuation of the previously constant Kahn titer in an elevated range, 2 showed primary diminution and

secondary elevation of titer, most marked in the Kahn but also evident in the other flocculation tests; one, after a previously negative reaction, showed a definite rise of titer, evident in all tests and comparable to that seen in the rabbit injected with flocculate from human syphilitic serum.

3. Of 2 rabbits treated with heated autogenous serum, both exhibited elevation of the Kahn titer during the first five weeks.

4. Two rabbits were treated with aqueous extract of rabbit tissues. One showed no definite serologic change. The other showed a rising Kahn titer but was found to have a splenic lesion, possibly a granuloma due to the injected lipids.

5. Two rabbits were treated with a lipid extract of rabbit tissues; one showed a very slight transient increase of titer, most evident in the Kahn test, and the other exhibited an incidental lesion, Echinococcus cyst, which is believed to have affected the serologic results.

6. Two rabbits were treated with a peptic metaprotein from "lipid free" rabbit tissues, and each showed a transient increase of titer most evident in the Kahn test but of doubtful proportions.

#### CONCLUSIONS

While the evidence brought forward may not be entirely convincing, it is compatible with, and is best explained by, the conception that reagin is a closely related group of antibodies and not a single chemically constant compound, and that, while the primary serologic change in syphilis may be the formation of antibodies to *T. pallidum*, the potential or actual occurrence of positive or doubtful reactions with lipid antigens in the absence of syphilis results from the formation of antibodies to the "ubiquitous lipid," either from some other infectious agent or from the tissues of the host, dependent upon the liberation of lipid haptens and protein derivatives capable of activating them.

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## VITAL CAPACITY STUDIES IN THE AGED

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SINCE 1846 the estimation of the vital capacity has been used as a measure of respiratory efficiency. Various standards and tables have been set based on occupation, weight, surface area, standing height, sitting height, and chest circumference. These standards were needed for comparison, since differences were found in the ability of men with the same vital capacity to resist dyspnea. In 1846 Hutchinson<sup>1</sup> in his work with the spirometer used the standing height as the standard of comparison, the vital capacity varying directly with the standing height. Peabody and Wentworth<sup>2</sup> also used standing height measurements; they divided people into three groups, according to height, and made average vital capacity readings for each group. Dreyer<sup>3</sup> and West<sup>4</sup> used surface area standards in their work. Dreyer<sup>3</sup> divided people into three groups, based on physical fitness. His vital capacity figures varied from high in the active athlete and laborer group to low in those with sedentary occupations. Training, sex, and occupation all played a part in the vital capacity.

Most of the work with vital capacity has been done on adults. Observations made on the aged have been only incidental and were based on relatively few cases. Hutchinson<sup>1</sup> found an increase in vital capacity with age up to 30 years, and a definite decrease after 50 years. Loomis<sup>5</sup> stated that with advancing years the vital capacity decreased with tolerable regularity, about 1.5 cubic inches each year. He reported that a healthy adult exhaled about 1,340 cubic inches of carbonic acid per hour; in men between 60 and 80 years the amount fell to 930 cubic inches, and in very old men it diminished to 670 cubic inches. Charcot<sup>6</sup> found that the functions of the respiratory apparatus were weakened in old age, the amount of the carbonic acid exhaled decreased, the number of inspirations increased, and the vital capacity of the lungs was reduced. Myers<sup>6</sup> studied the vital capacity of 88 women and 118 men, 66 years of age and over. The average vital capacity in men decreased with age from 74 per cent to 53 per cent; in women it decreased from 52 per cent to 44 per cent. Pratt<sup>7</sup> studied the influence of age on the vital capacity of 100 normal men in the second to the eighth decade of life. He reported a maximum capacity in the third decade, after which it slowly decreased. Bowen<sup>8</sup> studied the vital capacity of 184 people between 15 and 85 years, of whom 64 were over 50 years. He found the greatest drop between 50 and 60 years. Lemon and Moersch<sup>9</sup> stated that age had little effect before 50 or 60 years, and only a variable effect after that age. The decrease in vital capacity, if present, accompanied the decrease in physical fitness occurring at this period of life. The decrease was due not so much to age as to the degenerative changes and to the changes in height and weight which occurred in this age group. Levy<sup>10</sup> reported his studies

on the vital capacity of 110 men and 71 women, 60 to 94 years of age. In men the average vital capacity decreased from 2,980 c.c. at 60 to 65 years to 2,350 c.c. at 86 to 94 years. In women the average vital capacity decreased from 1,985 c.c. at 60 to 65 years to 1,460 c.c. at 86 to 92 years.

TABLE I

| AGE   | NUMBER OF MEN | AVERAGE VITAL CAPACITY |                      |
|-------|---------------|------------------------|----------------------|
|       |               | C.C.                   | PERCENTAGE OF NORMAL |
| 40-44 | 4             | 3,150                  | 79                   |
| 45-49 | 7             | 3,200                  | 80                   |
| 50-54 | 50            | 3,000                  | 75                   |
| 55-59 | 73            | 3,000                  | 75                   |
| 60-64 | 126           | 2,900                  | 72.4                 |
| 65-69 | 168           | 2,750                  | 70.6                 |
| 70-74 | 171           | 2,700                  | 70.2                 |
| 75-79 | 100           | 2,650                  | 69.2                 |
| 80-84 | 36            | 2,600                  | 68.8                 |
| 85-89 | 6             | 2,500                  | 68.1                 |
| 90-94 | 3             | 2,400                  | 62                   |

The vital capacity studies in this report were made on 744 men, 40 years of age and over. These men were inmates of the New York City Farm Colony, a home for the aged. The test was done in the afternoon hours, between meals, and in the different dormitories, which are situated on the ground floor and on the second floor. The men rested on the side of their beds for thirty minutes to rule out any possible dyspnea which might be due to walking or to climbing the one flight of stairs. Each man was given three trials at the spirometer, and the highest figure was taken as his normal. Weight and sitting height determinations were made on each man. The vital capacity readings were compared with the weight and sitting height, and the tables of Myers<sup>9</sup> were used to determine the percentage of normal.

Table I gives the average vital capacity and percentage of normal by five-year periods. There is a decrease in the average vital capacity from 3,150 c.c. at 40 to 44 years to 2,400 c.c. at 90 to 94 years. The decrease in percentage is from 79 to 62. The average vital capacity of the group as a whole was 2,780 c.c., 71 per cent of normal. Our determinations agree closely with those of Levy.<sup>10</sup>

#### DISCUSSION

Vital capacity is the sum total of tidal, supplemental, and complementary air; it is the maximum expiration of air as measured by a spirometer after the deepest inspiration. The vital capacity decreases with advancing age. The decrease is due to physiologic conditions that occur in the aging body. Degenerative changes are the most important. The lung tissue becomes less elastic, the thoracic cage is more fixed, and the muscular power diminishes.

Pulmonary pathology decreases the vital capacity. In 5 patients with pulmonary fibrosis and silicosis, the average vital capacity was 2,040 c.c., 50 per cent of normal. The figures for this group would have been lower if we had included several men whose attempts to do the test were not accurate. The first attempt at the spirometer brought on an attack of coughing and dyspnea, and further trials had to be abandoned. In 27 men with bronchiectasis the average



vital capacity was 2,130 c.e., 55 per cent of normal. Pulmonary tuberculosis (30 men) and pulmonary emphysema (31 men) gave a similar picture, an average vital capacity of 2,400 c.e., 62 per cent of normal. The patients with pulmonary tuberculosis had the productive type of lesion, with involvement of one or both apices or upper lobes.

Blood pressure variations had no effect on vital capacity with the exception of the group, with a systolic pressure of 200 mm. and over. This group had an average vital capacity of 2,400 c.e., 62 per cent of normal. Seventy men with heart murmurs, systolic murmurs at either the mitral or aortic areas, and 27 men with auricular fibrillation had normal vital capacity readings. Physical examination revealed no evidence of decompensation in these cases.

Vital capacity studies to be of use must be interpreted correctly. A single reading is not as accurate as a series of readings; a fall in vital capacity is important. A normal vital capacity does not denote the absence of pulmonary pathology; a low vital capacity is of significance. A vital capacity of 90 per cent and over is considered normal.<sup>11</sup> With a vital capacity of 80 to 90 per cent, work can be done, but any increased strain will produce dyspnea. With a reduction in vital capacity to 40 to 70 per cent, only light work can be done, and a slight increase in effort will produce dyspnea. With a vital capacity of 40 per cent or less, patients are usually dyspneic even without effort. Of the 744 patients 40 years of age and over, 11 per cent had a vital capacity of 90 per cent and over; 45 per cent had readings between 70 and 90 per cent; 43 per cent had readings between 40 and 70 per cent; and 2 per cent had readings under 40 per cent. It can be concluded that the older age group can do light or moderate work, but an increase in effort would produce dyspnea.

#### CONCLUSIONS

1. Vital capacity determinations were made on 744 men, 40 to 94 years of age. There was a decrease in vital capacity from 3,150 c.e. (79 per cent of normal) at 40 to 44 years to 2,400 c.e. (62 per cent) at 90 to 94 years. The vital capacity for the group as a whole was 2,780 c.e. (71 per cent). The causes of the decreased vital capacity are loss of elasticity of the pulmonary tissue, the lessened mobility of the thoracic cage, and diminished muscular power.

2. Pulmonary fibrosis, pulmonary tuberculosis, pulmonary emphysema, and bronchiectasis gave vital capacity readings below normal for their age groups.

3. Compensated heart cases gave normal vital capacity readings. Lowered figures were found in men with blood pressure of 200 mm. and over.

4. A normal vital capacity does not denote the absence of pulmonary pathology; a low vital capacity is significant.

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333 EAST 80TH STREET

## PULMONARY ASPERGILLOSIS\*

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ALTHOUGH mycotic infections of the lungs have been recognized for decades, they are still very interesting and unusually hard to diagnose because of their relative rarity. Hughes Bennett in 1842 reported the first case on record. Cases of pulmonary aspergillosis reported in the nineteenth century were made by Virchow, Dieulafoy, Chautemesse, and Widal.<sup>1</sup>

The species of the genus *Aspergillus* are predominantly saprophytic, but some are quite pathogenic. Because of its omnipresence, universal infection would undoubtedly occur were not the vast majority of this genus nonpathogenic.

Aspergillosis is produced by a moldlike fungus with a basal stem and stalk supporting a spore-bearing head. This organism seems to have a definite affinity for the pulmonary system,<sup>2,3</sup> although other organs may be attacked, especially the kidneys of experimental animals.

An interesting side light on this disease is given by C. W. Dodge:

The natives of Watusi make use of the pathogenicity of some species of *Aspergillus* in an interesting way, according to Mattlet (1924). When they wish to wreak vengeance on some one, they exhume a corpse of a person recently dead of a pneumomycosis, remove the lungs, desiccate them, and mix the powder in a banana beer. Evidently the *Aspergillus* is not killed by this process, and the recipient wastes away with the Aspergillosis, without suspecting the cause.<sup>2</sup>

### CASE REPORT

H. B. G., a white male, aged 40 years, a bookkeeper by occupation, who first came under observation on April 22, 1940, had a productive cough and fever. In August, 1939, he began to run an elevated temperature, and developed a yellow tinge of the skin. He had had a cough for several years, and two months before the present examination he had noticed pain in the chest. X-ray examination (Fig. 1) of the chest on August 6, 1939, revealed

\*From the Woodmen of the World War Memorial Hospital, San Antonio.  
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numerous scattered areas of infiltration throughout the middle and upper lobes of the right lung, especially marked in the first and second interspaces, while the mediastinum was displaced to the right. The left lung showed scattered infiltration throughout the entire upper lobe and the upper half of the lower lobe. Diffuse fibrosis was present throughout the left lung, especially marked in the upper lobe. Both hilar shadows were increased in density, while numerous circumscribed areas of rarefaction were present in both lung fields. Pulmonary tuberculosis was diagnosed from this x-ray picture, although the sputum was negative for tubercle bacilli. The patient entered the Texas State Tuberculosis Sanatorium on October 18, 1939. Many sputum examinations before entrance to the Sanatorium were negative for tubercle bacilli, as were numerous ones made during his six months' stay at this institution.



Fig. 1.—X-ray examination on Aug. 6, 1939, revealed numerous scattered areas of infiltration throughout the middle and upper lobes of the right lung. Areas of infiltration were present throughout the left upper lobe and the upper part of the lower lobe. Numerous areas of rarefaction were present in both lung fields.

Lipiodol injection of the bronchial tree revealed evidence of the presence of bronchiectasis, while a tentative diagnosis of cystic disease was made from the scattered areas of rarefaction observed in the x-ray examination.

The patient's family history was irrelevant. When a small child, he had measles, mumps, pertussis, and chickenpox. In 1918 he had influenza, and on one or two occasions since that date he has had repeated attacks. He had malaria in 1923 and a transient attack of asthma in August, 1938.

In an effort to determine the possible etiology of the asthmatic attack, the patient was referred to the allergist, Dr. B. H. Reinarz. He reported the patient to be sensitive to the following allergens: *Inhalants*: wool, mohair, and sterile mycelia. *Foods*: beef, corn, lamb, mutton, pork, veal, chocolate, goat's milk, and cow's milk.

Physical examination of all systems was negative, except as will be noted. There was blanching and thickening of the mucous membrane throughout the nose, with haziness of the sinuses, that of the left maxillary being especially marked.

Inspection of the chest showed the thorax to be poorly nourished, with all the bony landmarks prominent. There was a yellowish tinge to the skin. Decreased expansion was observed over the upper half of each hemithorax, there being a more marked restriction of expansion on the left. Increased tactile fremitus was present over the upper lobe of the left lung. Impaired resonance was elicited upon percussion of the right chest from the apex to the second rib anteriorly, and from the apex to the spine of the scapula posteriorly. Impaired resonance was present over the anterior portion of the left chest from the apex to the fifth rib, and from the apex to the angle of the scapula posteriorly. From auscultation the presence of harsh breath sounds was noted over the apex of the right lung anteriorly and posteriorly. The whispered voice was increased in intensity over the same area, but no râles were present. Increased intensity of the breath sounds was noted from the left clavicle to the fourth rib anteriorly, and over the suprascapular region posteriorly. Fine and medium moist râles were observed in these same areas.



Fig. 2.—X-ray examination on April 22, 1940, showed some clearing of the infiltration in both lungs since the last examination. Fibrosis was marked in both lung fields.

X-ray examination on April 22, 1940 (Fig. 2) revealed a decrease in the degree of the infiltration observed in the right lung on previous examination. Fibrosis was still marked especially in the middle and upper lobes. The mediastinum was displaced to the right. There had been slight clearing of the infiltration in the left lung. Scattered areas of rarefaction were still present in both lungs.

The urine was negative, except for an occasional pus cell; the erythrocyte count was 3,930,000; the leucocyte count was 7,000; the hemoglobin 85 per cent (Dare); polymorphonuclear leucocytes 65 per cent; lymphocytes 20 per cent; monocytes 10 per cent; eosinophiles 5 per cent; Kahn test negative; sedimentation rate 5.5 per cent, 14.5 per cent, 43 per cent; Weltmann's coagulation band 3. Ten sputum specimens were negative for tubercle bacilli. Cultures of the sputum on Sabouraud's glucose agar resulted in a growth of *aspergillus*, whose cultural characteristics are as follows: Milk coagulated, then liquefied; broth, large whitish surface colony; Sabouraud's and Krainsky's agar, tough, dull, yellowish-white growth; carrot



Fig. 3A.—Slide culture of the aspergillus showed mycelial strands from which branched stalks bearing conidia clustered about the conidiophore.

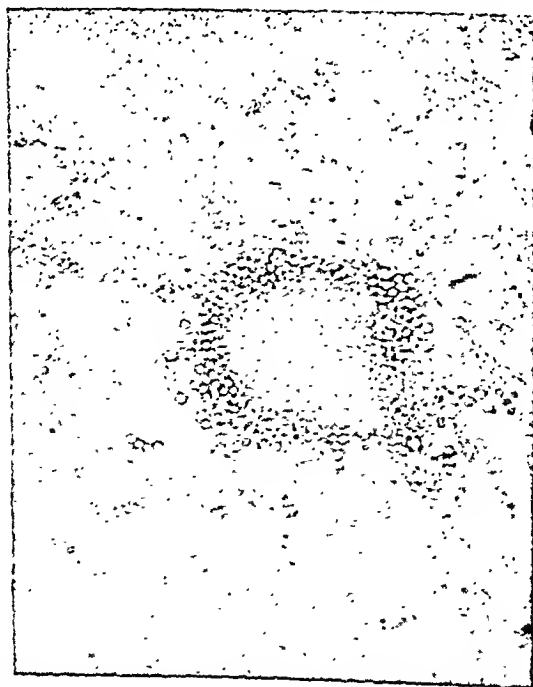


Fig. 3B.—Higher magnification of the slide culture revealed the structural arrangement of the spore-bearing stalk.

infusion agar, colonies small, round, and spinose; gelatin stab, white, cottonish surface growth. Acid but no gas was formed in dextrose, saccharose, maltose, and lactose.

Microscopic examination revealed a gram-negative nonacid-fast organism, exhibiting a branching mycelium with a basal stem and a stalk which terminated in a bulbous enlargement or conidiophore around which were clustered numerous conidia (Figs. 3A and 3B). The conidia ranged in size from one-third to one-half the size of an erythrocyte.

Intravenous injection of one-half of a forty-eight-hour agar slant culture of the aspergillus caused the death of a grown rabbit in three days. Post-mortem examination of the rabbit showed large hemorrhagic areas throughout both lungs, liver, and spleen, with petechial hemorrhages present in the cortex of the kidneys. Cultures from the various organs were planted on Sabouraud's agar, and a pure culture of the organism was recovered from the spleen.

From these findings a diagnosis of pulmonary aspergillosis was made, and the patient was placed upon iodide therapy. Potassium iodide was given orally in increasing doses until 40 drops were given three times daily. The patient's symptoms have disappeared after nine months' treatment and he has gained 45 pounds in weight.

#### COMMENTS

It has often been emphasized that aspergillosis has a distinct relationship to certain occupations. This is most commonly observed among bird fanciers<sup>10</sup> and pigeon stuffers<sup>11</sup> who feed the birds from their own mouths. This occupation affords a double hazard in the infected birds and the contaminated grain. Other occupations in which there is prolonged exposure to grain dust, such as occurs in farming, milling, threshing, and sponge cleaning afford ample opportunity for infection with this organism.<sup>1, 3</sup> It is likely that often repeated massive dosage is the chief factor in the pathogenesis of this disease. Asymptomatic cases have been found upon routine examination of iron miners in certain localities.

The concomitant occurrence of aspergillosis and other pulmonary diseases has prompted many investigators to advance the theory that aspergillosis does not occur in man unless the local or constitutional resistance is lowered. "Secondary aspergillosis" has been reported to complicate pulmonary tuberculosis, primary carcinoma,<sup>9, 13</sup> and other pulmonary conditions. Lowering of the general resistance by such diseases as uncontrolled diabetes, carcinoma, dysentery, and enteritis predisposes to the infection of the lungs with aspergillosis.

Lapham<sup>4</sup> in 1926 reported ten cases of primary aspergillosis which she divided into two groups, the wet or parenchymatous and the dry or interstitial types. However, the classification most often employed recognizes a superficial and a deep form. In the superficial type aspergillary catarrh and bronchitis are included. The deep type produces necrosis, and caseation of the lung tissue occurs. This organism shows a high<sup>4</sup> and constant virulence for laboratory animals. Large doses of the spores intravenously cause death of the animal in one or two days. The lesions produced by this massive infection showed only hemorrhagic and necrotic areas. Smaller doses cause death of the animal in a week or ten days. In studying these tissues Henrici noted that tubercles resembling those produced by tuberculosis resulted.<sup>5</sup>

The onset of the disease is insidious and may be manifested only by cough, asthmatic attacks, and anorexia. Later the cough is increased and the sputum

becomes tenacious and may be dotted with black specks<sup>14</sup> which are conidia. Night sweats, an evening rise in temperature, loss of weight, and hemoptysis occur as the disease progresses. Massive hemorrhages may be the first symptom that causes the patient to seek medical advice. In the asthmatic type progressive dyspnea is a distressing symptom. Pleurisy may be the most outstanding symptom as the patient relates his history.

Cavitation and destruction of the lung tissue are slow, and the course is protracted. However, when mixed infection of the diseased tissue occurs, the progression of the disease may be much more rapid.

As with other pneumomycoses, there is no characteristic x-ray picture. Involvement of the bases and hilar regions is most common. Limitation of the involvement to these areas is by no means constant.<sup>16</sup> Increased hilar shadows with radially distributed increased markings are said to be one of the earliest pictures presented by this disease. The x-ray may resemble bronchopneumonia in the early stage of resolution.<sup>3</sup>

Physical examination may reveal limited expansion of the chest, most marked on the side with the greater degree of involvement. Coarse and fine râles may be observed upon auscultation over the bases or hilar areas.

Diagnosis of aspergillosis must be made only when all other possible etiologic factors are ruled out. The finding of the aspergillus organism in the sputum is not sufficient evidence for making such a diagnosis. Culturing the organism, the reproduction of the lesion by means of animal inoculation, and the reisolation of the fungus are important factors in clinching the diagnosis. The presence of agglutinins in the patient's serum and positive skin reaction to stock or autogenous vaccines are additional aids in the diagnosis, although their value is questioned by some authorities.

In the treatment of pneumomycoses in general, a multitude of agents have been used. The same is true of the treatment of aspergillosis. The time-honored remedy employed in the condition is potassium iodide;<sup>12</sup> other medicaments often used are the inhalation of ethyl iodide,<sup>1</sup> neoarsphenamine intravenously, intramuscular bismuth, sodium iodide intravenously,<sup>15</sup> and the roentgen ray. Of course, the presence of concomitant pulmonary tuberculosis contraindicates the use of iodides. It is easily seen that there is no "specific" drug.

#### SUMMARY

1. Pneumomycosis should be considered in any patient with pulmonary symptoms.

2. A case of pulmonary aspergillosis was presented with a discussion of the mode of infection, pathology, symptomatology, x-ray picture, diagnosis, and treatment.

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## THE PERCUTANEOUS POTENCY OF PROGESTERONE\*

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IT HAS been known for some time that estrogens and androgens are absorbed through the skin of the experimental animal and produce systemic effects.<sup>1-3</sup> Various workers have made clinical observations demonstrating that the same effect is obtained in the human being.<sup>1, 9-12</sup> Unfortunately, both estrogens<sup>1, 2</sup> and androgens<sup>7, 8</sup> are much less potent when given percutaneously in an ointment than when given parenterally. Investigations on the percutaneous effectiveness of progesterone are limited to a statement by Zondek that in the rabbit it is less effective by this route than by subcutaneous injection.<sup>2</sup>

After parenteral administration progesterone is relatively rapidly absorbed, metabolized, and excreted. It resembles estrone or testosterone in this respect. The estrogens and androgens, however, may be given parenterally in the esterified forms (e.g., estradiol dipropionate and testosterone propionate) which are relatively slowly absorbed and have a prolonged effect. Thus the patient may be given adequate therapy with treatment at infrequent intervals. Progesterone, however, has no active esterified form; consequently, the patient should be treated frequently, probably every day, to obtain the best clinical response. The indications, if any, for progesterone therapy and the inadequacy of the dosages commonly employed are not pertinent to this discussion.

Since percutaneous application of progesterone would be a convenient method for daily treatment, it was decided to investigate the percutaneous potency of progesterone. A quantitative procedure such as this was not

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feasible in the human being; accordingly, the rabbit was used. We realize, of course, that skin absorption of progesterone is not necessarily the same in the rabbit as in the human being. Therefore, the results obtained in the rabbit can only be considered as indicative of what might happen in the human being.

#### PROCEDURE

Thirty-seven virgin immature rabbits were used in this study. All were primed with 0.002 mg. estrone on the first and third days of the experiment. Five animals were given no further treatment and served as controls. The remaining animals were given progesterone\* daily, starting on the fifth day. All animals were killed on the tenth day. The uteri were dissected out, weighed, fixed in Bouin's fluid, and prepared for microscopic study. The progesterone-treated animals were divided into eight groups (Table I). One group was treated parenterally, and the remainder were treated percutaneously. The substance for parenteral administration was applied to an area on the back from which the hair was clipped.

TABLE I

| TREATMENT<br>(DAILY DOSE)        | NO. OF<br>ANIMALS | AVERAGE BODY<br>WEIGHT<br>(GM.) | AVERAGE<br>UTERINE<br>WEIGHT<br>(GM.) | MCPHAIL<br>RATING<br>(AVERAGE) |
|----------------------------------|-------------------|---------------------------------|---------------------------------------|--------------------------------|
| Control (primed)                 | 5                 | 667                             | 0.364                                 | 0                              |
| Subcutaneously 0.1 mg. (oil)     | 5                 | 683                             | 0.862                                 | 2.6                            |
| Percutaneously 0.1 mg. (oil)     | 2                 | 630                             | 0.383                                 | 1.0                            |
| Percutaneously 0.3 mg. (oil)     | 4                 | 654                             | 0.456                                 | 1.25                           |
| Percutaneously 0.6 mg. (oil)     | 4                 | 694                             | 0.765                                 | 2.37                           |
| Percutaneously 0.1 mg. (alcohol) | 5                 | 684                             | 0.561                                 | 1.4                            |
| Percutaneously 0.2 mg. (alcohol) | 5                 | 763                             | 0.590                                 | 1.6                            |
| Percutaneously 0.4 mg. (alcohol) | 5                 | 549                             | 0.995                                 | 3.0                            |
| Percutaneously 0.6 mg. (alcohol) | 2                 | 652                             | 1.697                                 | 4.0                            |

In all cases the progesterone was given in 0.1 c.c. of solvent. Alcohol was used as well as oil for percutaneous application, since it has been demonstrated that both estrogens and androgens are more potent when applied percutaneously in this solvent.<sup>2, 6, 7</sup>

The progestational responses of the uteri were graded according to the histologic method of McPhail.<sup>13</sup>

#### RESULTS

The mean uterine weights and progestational responses, as well as the treatment details for each group, are presented in Table I.

The number of animals in each group is adequate for an estimation only of the potency of progesterone when given by different methods. It is obvious, however, that in an oil solvent progesterone is not very effective by the percutaneous route. With 0.1 mg. the uteri were not significantly larger than those of the castrate controls, and histologically they showed only slight stimulation. The effect was only slightly greater with 0.3 mg., and even 0.6 mg. produced uteri which were smaller and had a lower histologic rating than 0.1 mg. parenterally.

\*The progesterone used in this study was furnished by Schering Corporation.

The alcohol solvent definitely increased the effectiveness of the percutaneous progesterone. However, even in this solvent the percutaneous route was less effective than the parenteral, 0.1 and 0.2 mg. both produced smaller uteri and lower histologic ratings than did 0.1 mg. parenterally. However, 0.4 mg. percutaneously produced a somewhat greater effect than 0.1 mg. parenterally.

#### DISCUSSION AND SUMMARY

These data indicate that progesterone is less effective by the percutaneous route than by the parenteral method. With oil as the solvent it is less than one-sixth as potent. With alcohol as the solvent it is less than one-half, but more than one-fourth, as potent percutaneously as parenterally.

Any clinical application of these data must rest on the assumption that skin absorption of progesterone is the same in man as in the rabbit. The validity of this assumption can only be tested by clinical experience. Assuming, however, that the skin of the human being would react in a manner similar to that of the rabbit, it is obvious that very large doses of progesterone would have to be given to obtain a satisfactory clinical response.

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# THE DETERMINATION OF THE DOSAGE-MORTALITY RATIO OF PENTOTHAL SODIUM WITH SULFANILAMIDE\*

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WITH the increasing use of sulfanilamide in the fields of medicine and surgery, the anesthetist is frequently called upon to administer an intravenous anesthetic, particularly pentothal sodium (sodium ethyl 1-methyl butyl thiobarbiturate), to a patient who has been receiving sulfanilamide (para-aminobenzenesulfonamide) or one of its derivatives. Adriani<sup>1</sup> has suggested that the sulfanilamide-barbiturate sequence may be unwise in human therapy.

In his experiments on rats previously treated with 0.5 to 1.0 Gm. per kilogram of sulfanilamide, a dose which Nelson<sup>2</sup> describes as fatal for rabbits, susceptibility to anesthetic doses of the barbiturates was increased. The thiobarbiturates were the worst offenders in this respect.

The present study was undertaken with the purpose of determining whether the toxicity of pentothal sodium is increased by previous administration of sulfanilamide in therapeutic doses (0.15 Gm. per kilogram per day). The above dose agrees fairly well with that recommended by Long and Bliss,<sup>3</sup> who used 0.11+ Gm. of sulfanilamide per kilogram for human therapy.

The animals used were white Wistar strain rats, between the ages of six weeks and three months, weighing approximately 150 Gm. Sulfanilamide was prepared by dissolving the crystals in sterile distilled water to make a 1 per cent solution. Intraperitoneal injections of 0.15 Gm. of sulfanilamide per kilogram of body weight were then made daily for seven days. On the eighth day pentothal sodium (2.5 per cent solution) was given intraperitoneally to eight groups of 10 rats each, each group receiving a different dose. The doses ranged from 50 to 110 mg. per kilogram. As a control eleven groups of 10 rats each were given 40 to 140 mg. per kilogram of pentothal sodium, having had no previous treatment with sulfanilamide.

Computations of the  $L. D_{50}$  were carried out according to the method of Bliss,<sup>3</sup> using the equation  $\log L. D_{50} = \bar{x} + s(5 - \bar{y})$  for the determination of the logarithm of the median lethal dose, and the equation  $V(\log L. D_{50}) = s^2 \left( \frac{(5 - \bar{y})^2}{(wxy)} + \frac{1}{S(w)} \right)$  for the standard error of deviation.

From the data in Table I the log of  $L. D_{50}$  for pentothal sodium alone, and for pentothal sodium with sulfanilamide has been computed, with their standard

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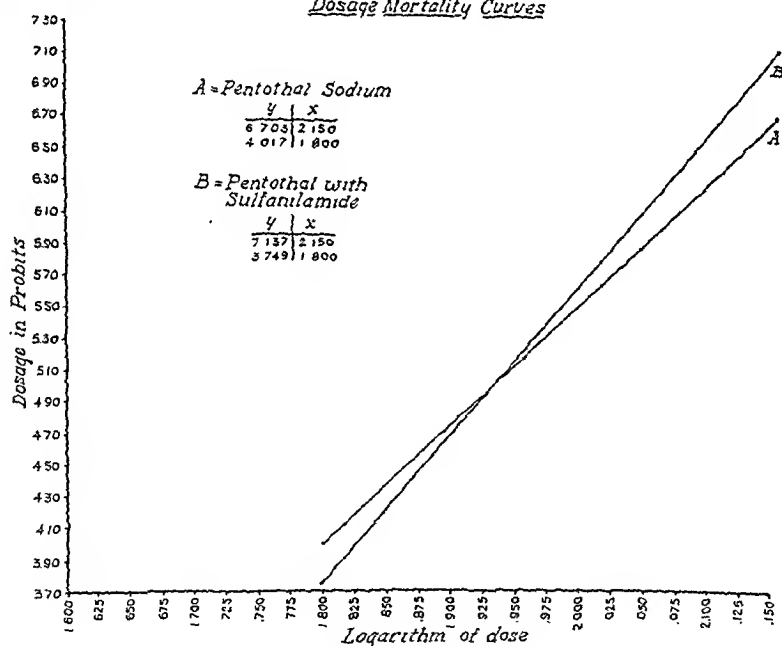
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errors. Graph 1 shows the computed curves for pentothal sodium and pentothal sodium with sulfanilamide.

TABLE I  
TOXICITY OF PENTOTHAL SODIUM AND OF PENTOTHAL SODIUM SUBSEQUENT  
TO SULFANILAMIDE THERAPY

| NO. | PENTOTHAL SODIUM |                              | PENTOTHAL SODIUM WITH<br>SULFANILAMIDE (15 MG.) |                              |
|-----|------------------|------------------------------|---|------------------------------|
|     | DOSE IN MG.      | NUMBER OF RATS<br>DEAD/TOTAL | DOSE IN MG.                                     | NUMBER OF RATS<br>DEAD/TOTAL |
| 1   | 40               | 0/10                         |   |                              |
| 2   | 50               | 1/10                         | 50  | 0/10                         |
| 3   | 55               | 1/10                         |   |                              |
| 4   | 60               | 1/10                         | 60  | 1/10                         |
| 5   | 65               | 2/10                         |   |                              |
| 6   | 70               | 3/10                         | 70  | 2/10                         |
| 7   | 75               |                              | 75  | 3/10                         |
| 8   | 80               | 3/10                         | 80  | 4/10                         |
| 9   | 90               | 5/10                         | 90  | 6/10                         |
| 10  | 100              | 6/10                         | 100   | 7/10                         |
| 11  | 110              | 9/10                         | 110   | 9/10                         |
| 12  | 140              | 10/10                        |   |                              |
|     | $L.D_{50}$       | $84.85 \pm 4.00$             |   | $85.2 \pm 6.56$              |

Dosage Mortality Curves



Graph 1.

## RESULTS

By the above method the median lethal dose ( $L.D_{50}$ ) for pentothal sodium was computed to be  $84.85 \pm 4.00$  mg. per kilogram. This result is in fair agreement with that of Pratt and his co-workers,<sup>5</sup> who found the  $M.L.D.$  for pentothal sodium in rats to be 80 mg. per kilogram, although they do not state how they arrived at this figure. The median lethal dose ( $L.D_{50}$ ) for pentothal

sodium in rats which had previously received therapeutic doses of sulfanilamide was calculated to be  $85.21 \pm 6.56$  mg. per kilogram. By comparison of these median lethal doses (L. D.<sub>50</sub>) there is no significant difference in the toxicity of pentothal when given to rats which have received therapeutic doses of sulfanilamide.

### CONCLUSION

These data justify the conclusion that sulfanilamide in therapeutic doses does not increase the toxicity of pentothal sodium given subsequently to white rats, does not render the animals more susceptible to the effects of pentothal sodium, nor do the two drugs exert synergistic action, at least of a harmful nature, with this dosage.

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## IMMUNITY STUDIES OF CRYPTOCOCCUS HOMINIS (TORULA HISTOLYTICA) IN MICE\*

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A REVIEW of published reports (see references†) demonstrates that a mild form of hypersensitivity, usually revealed by skin reactions, occurs in patients infected with torula. Tests for serum antibodies are inconclusive. This study was undertaken to determine evidence of immunity against torula in a highly susceptible animal, such as the white mouse.

### MATERIALS AND METHODS

The *Cryptococcus hominis* strain used for the study was isolated in 1935 from a patient with torular meningitis. Growth on glucose agar was abundant in forty-eight hours at 37° C. Cultures from slants of this medium were suspended in normal saline, and the concentrations were estimated with a hemocytometer. Other methods for standardization of suspension were unsatisfactory. The suspensions were heated for fifteen minutes at 100° C. The torula were killed by two to four minutes' exposure to this temperature. Aqueous

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extracts were obtained by Berkefeld filtration of old cultures (two and a half months) in peptone broth and synthetic medium.\* A concentrate of the synthetic medium was prepared by precipitation with four parts of acetone and reprecipitation from normal saline solution. The final volume was one-tenth that of the original filtrate. Serologic tests were made with torula treated by the Stoddard and Cutler method. To a flask containing a saline suspension of torula, 0.1N hydrochloric acid was added to a pH of 4. After the mixture digested for forty-eight hours at 10° C., the organisms were removed by centrifugation and washed free of acid. The torula were used as the agglutininogen and the neutralized acid extract was used as the precipitinogen for the demonstration of serum antibodies.

#### DETERMINATION OF THE MINIMUM LETHAL DOSE

Each of 18 mice received 300,000,000 live torula intraperitoneally. This dose was established by preliminary experiments. All the mice died within twenty-five days; 40 per cent died in eleven days; 50 per cent in twelve days; 70 per cent in fourteen days. Using twelve days arbitrarily as the time limit, and 50 per cent as the number of mice killed with a dose of 300,000,000 torula, an arbitrary M.L.D. was obtained with a range of error of 11 per cent.

#### IMMUNITY FROM INJECTIONS OF DEAD TORULA

Twenty-three mice were immunized by twelve injections of 30,000,000 heat-killed torula at three-day intervals. The mice were then infected with live torula as was an equal number of control mice. All nonimmunized mice were dead in sixteen days, and the immunized mice in seventeen days. Twelve control mice and six immune mice were dead after eight days. On the tenth day the ratio was 18:10; on the twelfth day, 22:15. In the immunized group the death rate from the seventh to twelfth days was approximately 50 per cent that of the nonimmunized mice.

The gross pathology at post mortem did not differentiate the immune mice from the control mice. In the histologic examination of the immune mice, the lungs varied from complete susceptibility, represented by a massive hemorrhagic pneumonia, to a moderate degree of immunity, represented by patches of proliferated cells and thickening of the alveolar walls by hyperplasia of the septal cells. Immune changes observed in the brain were: fewer and smaller cysts, fewer torula, and single torula without cyst formation. Some brains had plaques of proliferated cells where the pial vessels course through the cortex. Occasionally there was proliferation of the endothelium of the choroid plexuses.

In experiments performed with rats, pairs of immunized and control rats were killed at seven-day intervals following the infecting dose. Thus the evolution of the lesions, as well as the character of the changes, could be observed. The histologic immune process early consisted of cellular proliferation of the septal cells of the lung, reticulo-endothelial cells of the spleen and the liver, and connective tissue growths in the omentum forming irregular focal regions of compact cellular tissue. As these changes progressed, foreign body giant

\*Distilled water with 6 per cent ammonium lactate, 0.2 per cent dipotassium phosphate, and 0.5 per cent sodium chloride.

cells with torula appeared. Later, the fibrous tissue elements became more prominent, and loosely woven nodules of fibrous tissue in whorls with epithelioid cells formed. Langhans giant cells were numerous and in combination with the nodules resembled tuberculous foci. The immunity expressed itself mainly as a hastening of the stages of cellular proliferation with subsequent scar tissue reaction and disappearance of the torula.

#### IMMUNITY FROM INJECTION OF BROTH FILTRATE

The immunity obtained in these animals was comparable to that of the previous group, but less marked. Attempts to reveal soluble toxins gave negative results.

#### SENSITIZATION TO TORULA

Mice were infected with torula, each mouse receiving 25,000,000 organisms. After twelve, sixteen, and twenty days, tests for hypersensitivity by intradermal injections on the shaven abdomens gave no reactions. The test solutions were broth filtrate, filtrate of the synthetic medium, and the concentrate of the latter filtrate. Intratesticular injections also were reactionless. Skin tests on an immunized rabbit were likewise negative. Accordingly, either the time required for the development of sensitization was long or these animals were not susceptible to such a response to this strain of torula.

#### DETERMINATION OF HUMORAL IMMUNITY

Six intravenous injections of heat-killed torula were made in a rabbit at three-day intervals. One cubic centimeter of dilutions of immune and control sera was mixed with 1 c.c. of a suspension of torula prepared as described, and placed in a water bath at 37° C. The suspensions of torula were stable for four hours. At the end of one hour the agglutination titer was 1:180. After five hours it was 1:280, with 1:60 in the controls. Agglutination tests with three different control serums compared with saline controls proved there was no nonspecific agglutination.

#### SUMMARY

1. Injections of torula suspension killed by heat prolonged life and modified the histologic structure of the lesions in mice subsequently infected with torula.
2. No exotoxin or cutaneous hypersensitivity was demonstrated with this strain of torula.
3. Agglutinins were demonstrated in the serum of an immunized rabbit to a titer of 1:180.

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## THE EFFECT OF SPLENIC EXTRACTS FROM CASES OF ESSENTIAL THROMBOCYTOPENIC PURPURA ON THE PLATELETS AND HEMATOPOIETIC ORGANS OF RABBITS\*

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THE relation of the spleen to the production of essential thrombocytopenic purpura has for some time been a subject of much dispute among investigators. In 1915 Frank<sup>1</sup> suggested that the spleen was responsible for the formation of an agent which exerts a destructive action upon the megakaryocytes of the bone marrow. The assumption that this toxin is capable of involving the bone marrow as a whole was proposed by Minot<sup>2</sup> in 1917. Kaznelson,<sup>3</sup> who first removed the spleen in a case of essential thrombocytopenic purpura, believed that the thrombocyte reduction in the peripheral blood was brought about by an increased thrombolytic activity of the spleen. Torrioli and Puddu<sup>13</sup> showed injury of the megakaryocytes in bone marrow cultures after the application of concentrated doses of splenic extracts from cases of essential thrombocytopenic purpura.

The recent investigations of Troland and Lee<sup>15, 16</sup> demonstrate a marked depression of the circulating blood platelets in rabbits after intravenous injection of acetone extracts of splenic tissue taken from three patients operated on for essential thrombocytopenic purpura. In September, 1939, Pohle and Meyer<sup>9</sup> reported that acetone extracts of the spleen from three patients with essential thrombocytopenic purpura produced no effect on the platelet levels of injected rabbits. Major and Weber<sup>7</sup> injected extracts prepared from the spleens of two

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patients with essential thrombocytopenic purpura. The splenic extract from one patient produced an increase in the blood platelet count, while the extract from the second patient caused a moderate fall in the platelet level. Tocantins<sup>12</sup> was unable to demonstrate the presence of platelet-destroying substances in the spleen and urine of three patients with thrombocytopenic purpura. Following the injection of 45 c.c. of an acetone extract of a thrombocytopenic spleen into a rabbit, Hobson and Witts<sup>3</sup> reported a depression in the platelet count from 525,000 to 109,000 in thirty hours. Rose and Boyer<sup>10</sup> record studies which are confirmatory of the work of Troland and Lee.<sup>15, 16</sup> Because of the bearing of these experiments on the mechanism of the production of essential thrombocytopenic purpura, it seemed important to repeat them and to include in the study changes in the bone marrow and hematopoietic organs.

#### MATERIAL AND METHODS

Extracts were prepared from splenic tissue obtained from five patients with essential thrombocytopenic purpura and from six other control patients with splenic enlargement. Sufficient clinical and hematologic data are presented to establish the accuracy of the diagnosis of the cases.

##### CASE 1.—Patient With Essential Thrombocytopenic Purpura:

D. T., a white girl, aged 14 years, was admitted to the Illinois Research and Educational Hospitals on March 10, 1938, complaining of frequent attacks of epistaxis and excessive menstrual bleeding. Bleeding from the nose was first noticed in October, 1937, at which time she experienced a nosebleed which persisted for two days. Menstrual period in November, 1937, continued for nine days, and in December, for fourteen days.

Physical examination was essentially negative, except for numerous petechiae, especially over both lower extremities. The spleen was not palpable. Blood studies on entrance showed platelets 48,040, erythrocytes 3,680,000, hemoglobin 8.5 Gm., and a definite megakaryocytic increase of the sternal bone marrow. On March 19 the bleeding time was three minutes and the coagulation time (Howell's method) was ten minutes. There was no clot retraction at the end of twenty-four hours and the tourniquet test was positive. Blood studies on March 25 showed platelets 61,200, erythrocytes 2,930,000, hemoglobin 7.25 Gm., and megakaryocytic hyperplasia of the sternal bone marrow. Splenectomy was performed on April 11, 1938. The spleen weighed 157 Gm. Twenty-four hours after operation the platelet count was 72,300. The platelets increased progressively, and on April 18 (seven days after splenectomy) they numbered 386,400. Following splenectomy there has been no bleeding at any time.

##### CASE 2.—Patient With Essential Thrombocytopenic Purpura:

R. V., a white woman, aged 22 years, admitted to the Illinois Research and Educational Hospitals on June 24, 1938, had frequent attacks of epistaxis. Nasal hemorrhages commenced about Feb. 15, 1938, and usually persisted for about one-half hour. She continued to have two or three hemorrhages weekly up until June 1, when the attacks became more frequent and prolonged. Menstrual history was normal until June 12, 1938, and continued until July 1, with a marked increase in the bleeding. Physical examination revealed a moderate pallor of the skin and numerous bright red petechiae over chest, abdomen, back, and extremities. The spleen was palpable and the tourniquet test was strongly positive. Blood studies on entrance showed platelets 24,400, erythrocytes 2,400,000, hemoglobin 6.3 Gm., and marked increase of the megakaryocytes on sternal bone marrow examination. The coagulation time was five minutes and the bleeding time was eight minutes. There was no clot retraction at the end of twenty-four hours. Splenectomy was performed on June 30. The spleen weighed 270 Gm. Five hours postoperatively there were 136,200 platelets with many large forms. Twenty-four hours after splenectomy platelets numbered 185,400, and on July 11 (eleven days after surgery) the platelet count was 473,000.

## CASE 3.—Patient With Essential Thrombocytopenic Purpura:

F. R., a white woman, aged 21 years, first entered the Illinois Research and Educational Hospitals on Dec. 5, 1938, complaining of frequent attacks of epistaxis, bleeding from the gums, vagina, and rectum. She was in good health until the latter part of October, 1938, when she first noted a small amount of bright red blood after defecation. Shortly afterwards she began to have frequent attacks of epistaxis. The menstrual period during the first week of November was very *profuse* and continued for eight days. Physical examination revealed numerous petechiae distributed over both lower extremities. The spleen was palpable, and the tourniquet test was markedly positive. Blood studies on entrance showed a platelet count of 29,740, erythrocytes 4,970,000, hemoglobin 13.6 Gm., and a megakaryocytic hyperplasia of the sternal bone marrow. The coagulation time was eight minutes and the bleeding time was ten minutes. There was no clot retraction at the end of thirty hours. Splenectomy was performed on Dec. 14, 1938. The spleen weighed 132 Gm. Twenty-four hours after operation the platelet count was 171,400, and on December 19 the platelets numbered 314,700. Bleeding subsided completely following the splenectomy.

## CASE 4.—Patient With Essential Thrombocytopenic Purpura:

P. H., a white boy, aged 15 years, was admitted to the Illinois Research and Educational Hospitals on Sept. 12, 1939, complaining of epistaxis. Bleeding from the nose was first experienced in June, 1939, and was followed in a short time by persistent oozing of blood from the gums. Physical examination was essentially negative, except for numerous petechiae over the posterior aspect of the neck, cubital region, and chest. Blood studies on entrance showed platelets 40,000, erythrocytes 4,490,000, hemoglobin 13.0 Gm., and a definite megakaryocytic increase of the sternal bone marrow. The bleeding time was twenty minutes and the coagulation time was nine minutes. There was no clot retraction at the end of twenty-four hours. The patient remained in the hospital under observation for several months. During this time he had many severe attacks of epistaxis. Platelet counts as low as 11,000 were obtained. Splenectomy was performed on Dec. 21, 1939. The spleen weighed 290 Gm. Six hours after removal of the spleen the platelets numbered 76,000, and eight days postoperatively a count of 980,000 was obtained.

## CASE 5.—Patient With Essential Thrombocytopenic Purpura:

B. P., a white girl, aged 17 years, was admitted to the Illinois Research and Educational Hospitals on July 26, 1940. She had severe epistaxis, bleeding gums, purpuric spots, and bruised easily since December, 1938. At the time of entrance she was pregnant approximately seven months. Physical examination revealed red, spongy gums. The liver was palpable and the tip of the spleen could be felt. The bleeding was controlled, and the patient was allowed to go into labor spontaneously on Sept. 29, 1940. The puerperium was afebrile and uneventful. Blood studies on admittance to the hospital showed platelets 100,000, erythrocytes 3,120,000, hemoglobin 9.8 Gm., and a myeloid erythroid and megakaryocytic hyperplasia of the sternal bone marrow. The bleeding time was twelve minutes, the coagulation time was nine minutes, and the beginning clot retraction occurred at the end of one and one-half hours. She was prepared for splenectomy by the administration of blood, and on Oct. 23, 1940, the spleen was removed. Twenty-three days postoperatively blood studies revealed platelets 200,000, erythrocytes 5,910,000, and hemoglobin 17.5 Gm. Since splenectomy there has been no bleeding from the nose or gums.

Splenic tissue obtained from six control cases was used in the preparation of extracts. They included a normal spleen and cases of congenital hemolytic anemia, splenic vein thrombosis, chronic lymphatic leucemia, aplastic anemia, and Banti's syndrome.

The method of extraction used in this investigation has been described in detail by Troland and Lee.<sup>15, 16</sup> Suffice it to say that the macerated splenic tissue was placed in acetone. After a variable length of time the acetone was filtered off and then distilled. The sediment was dissolved in 100 c.c. of distilled water

and filtered. Extracts were stored in the refrigerator and injected within forty-eight hours after preparation. The type of acetone (commercial or reagent) used in the extraction seemed to have no influence on the results obtained. Table I contains the extracts prepared, type of acetone used in the extraction, weight of the splenic tissue, grams of the splenic tissue in a 20 c.c. dose of the splenic extract, and the length of time the tissue was allowed to remain in the acetone.

TABLE I  
SPLENIC EXTRACTS

| SPLENIC EXTRACTS                   | TYPE OF ACETONE | WEIGHT OF SPLENIC TISSUE IN GRAMS | TIME OF EXTRACTION IN ACETONE IN DAYS | GRAMS OF SPLENIC TISSUE IN 20 C.C. SPLENIC EXTRACT | GRAMS OF SPLENIC TISSUE IN 10 C.C. SPLENIC EXTRACT |
|------------------------------------|-----------------|-----------------------------------|---------------------------------------|--|--|
| E. 1. Thrombocyto-penic purpura    | Commercial      | 130                               | 253                                   | 26.0   | 13.0   |
| E. 2. Thrombocyto-penic purpura    | Reagent         | 140                               | 182                                   | 28.0   | 14.0   |
| E. 3. Thrombocyto-penic purpura    | Reagent         | 109                               | 35                                    | 21.8   | 10.9   |
| E. 4. Thrombocyto-penic purpura    | Reagent         | 82                                | 274                                   | 16.4   | 8.2  |
| E. 5. Thrombocyto-penic purpura    | Reagent         | 203                               | 30                                    | 40.6   | 20.3   |
| E. 6. Normal control               | Reagent         | 125                               | 19                                    | 25.0   | 12.5   |
| E. 7. Splenic vein thrombosis      | Reagent         | 114                               | 291                                   | 22.8   | 11.4   |
| E. 8. Congenital hemo-lytic anemia | Reagent         | 285                               | 51                                    | 57.0   | 28.5   |
| E. 9. Chronic lym-phatic leucemia  | Reagent         | 123                               | 91                                    | 24.6   | 12.3   |
| E. 10. Aplastic anemia             | Reagent         | 127                               | 61                                    | 25.4   | 12.7   |
| E. 11. Banti's syndrome            | Reagent         | 250                               | 18                                    | 50.0   | 25.0   |

In the present study the number of blood platelets was determined by the direct method. In some instances the indirect blood smear method was used to check the results obtained by the direct counts. The diluting fluid used consists of 0.6 Gm. sodium chloride, 0.2 Gm. sodium citrate, and 0.04 Gm. brilliant cresyl blue made up to 100 c.c. with distilled water. Blood was obtained from a stab wound of the marginal vein of the rabbit's ear. The blood was drawn to the 0.5 mark in a standardized erythrocyte pipette and diluted to the 101 mark with the platelet diluting fluid. After shaking for two minutes a certified counting chamber with improved Neubauer ruling was filled and allowed to stand for ten minutes. Counts were made with a high, dry (4 mm.) objective and a 12.5x ocular.

Normal rabbits, approximately 2 kg. in weight, were employed as the experimental animals. A new rabbit given no food for twelve hours previously was used for each experiment. Several control platelet counts were made prior to the injection of the splenic extracts. After administration of the extract, platelet counts were repeated every two to four hours for a period of twenty-four to forty-eight hours.

Bone marrow sections and bone marrow imprints, together with sections of the liver, lung, and spleen were obtained from seven animals. After injection of 20 c.c. of the extracts prepared from the essential thrombocytopenic purpura specimens, the rabbits were sacrificed at the maximal platelet count depression. Following the injection of a control extract (E. 7), the rabbit was sacrificed at the time interval required to produce maximal platelet drop with the essential thrombocytopenic purpura extracts. Specimens of the bone marrow, liver, lung, and spleen were also obtained from a normal untreated rabbit. Pieces of tissue were fixed in a solution of formaldehyde and in Zenker's fluid. After preparation of the sections they were stained by hematoxylin and eosin and Giemsa's stains.

On examination of the bone marrow sections the number of megakaryocytes per 1,500 nucleated bone marrow cells was determined. The bone marrow imprints were prepared by touching the cut end of the sternum to a glass slide. Each slide contained eight such touch smears of approximately equal size. In enumerating the megakaryocytes on the bone marrow imprints all megakaryocytes exclusive of the central portion of the imprint were counted. The central portion of the imprints were not included in the counts because of the deep stain which they assumed, preventing accurate cellular differentiation. The megakaryocyte counts of the imprints were determined by taking the average of eight imprints.

#### EXPERIMENTAL OBSERVATIONS

Splenic extracts prepared from the essential thrombocytopenic purpura specimens, Cases 1, 2, 3, 4, 5, will be designated as extracts (E) 1, 2, 3, 4, 5, respectively. After injection of 20 c.c. of E. 1, a depression in the platelet count was noted from the preliminary count of 500,000 to 325,000 within eight hours. Twelve hours after injection the platelet count was 450,000, and within twenty-four hours it had reached the preinjection level.

Injection of 20 c.c. of E. 2 produced practically the same result as that of E. 1. A depression in the platelets from the preinjection level of 492,000 to 315,000 was obtained within eight hours. Twenty hours later the platelet count was 440,000, and within twenty-four hours it reached the level determined prior to injection of the extract.

E. 3 differed from the previously mentioned extracts in that the maximal platelet depression was not reached until sixteen hours had elapsed. After injection of 20 c.c. of E. 3 the platelet count fell from a preinjection level of 485,000 to 315,000. The platelets returned to the preinjection level within twenty-four hours.

Following the injection of 20 c.c. of E. 4, a depression in the platelet count was noted from the preliminary count of 525,000 to 455,000 within twelve hours. At the end of twenty-four hours a count of 495,000 was obtained. The experiment was repeated, using 40 c.c. of E. 4. This dosage produced a drop in the platelet count from the preliminary level of 495,000 to 350,000 within twenty-four hours.

Injection of 20 c.c. of E. 5 caused a depression in the platelet count from a preliminary level of 500,000 to 405,000 within four hours. At the end of twenty-four hours the platelets numbered 485,000.

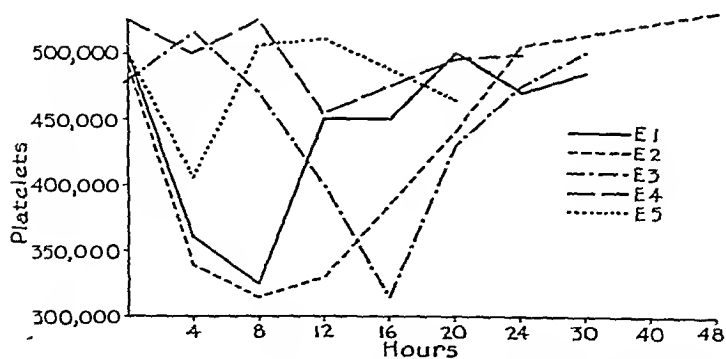


Fig. 1.—Platelet level following injection of 20 c.c. of thrombocytopenic extracts.

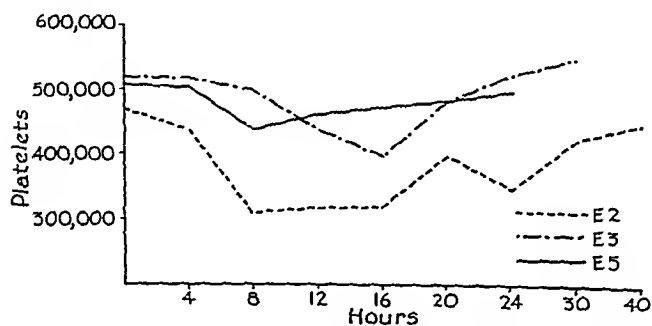


Fig. 2.—Platelet level following injection of 10 c.c. of thrombocytopenic extracts.

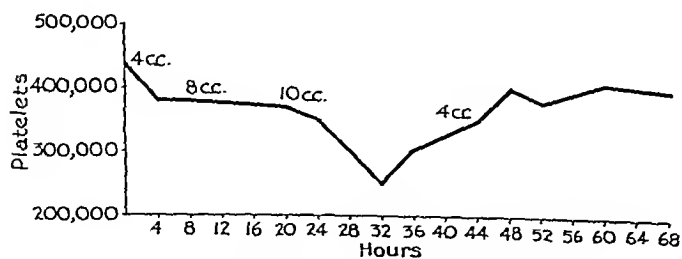


Fig. 3.—Determination of minimal effective dose.

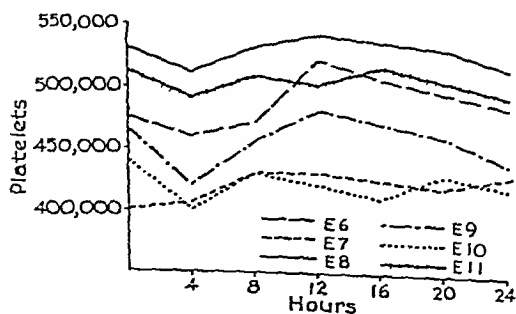


Fig. 4.—Platelet level following injection of control extracts.

The experiments were repeated using 10 c.c. of the splenic extracts, E. 2, E. 3, and E. 5. After the injection of E. 2 a depression of the platelet level resulted that was essentially the same as that produced by 20 c.c. of the extract.

Ten cubic centimeters of E. 3 produced a less marked depression of the platelets. The platelet count prior to injection was estimated at 520,000; sixteen hours later it was 400,000; and within twenty-four hours it had attained the preinjection level.

The injection of 10 c.c. of E. 5 was followed by a maximal platelet depression of 75,000 within eight hours.

In an attempt to determine the minimal effective dose of the extract, 4 c.c. of E. 2 were injected intravenously. A depression of 55,000 in the platelet count was obtained within eight hours. At this time 8 c.c. of the extract was injected. Twelve hours later the count dropped an additional 10,000 platelets. Then 10 c.c. of the extract was injected, and it was noted that a total maximal platelet depression of 185,000 was reached twelve hours after the injection of 10 c.c. of the extract.

Experiments using the control specimens were carried out with the same procedure employed in the cases of essential thrombocytopenic purpura. Twenty cubic centimeters of the extract (E. 6) prepared from the normal spleen were injected. A depression in the platelet count from 475,000 to 460,000 in four hours was noted. There was a return to the preinjection level within twelve hours. Other control specimens produced the following results:

After the injection of 20 c.c. of E. 7 (splenic vein thrombosis) no depression was noted. Twenty-four hours later the platelet count exceeded the initial count by 28,000. Twenty cubic centimeters of E. 8 (congenital hemolytic anemia) caused a drop of 22,000 platelets within four hours with a return to the preinjection level within eight hours. Following the injection of E. 9 (chronic lymphatic leucemia) a depression of 45,000 platelets within four hours was observed, with a return to the preliminary count within twelve hours. Ten cubic centimeters of E. 10 (aplastic anemia) produced a depression of 40,000 platelets within four hours. After the injection of 20 c.c. of E. 11 (Banti's syndrome) a drop of 22,500 platelets within four hours was noted.

Examination of the bone marrow imprints and sections revealed little, if any, change in the number of megakaryocytes of the rabbits injected with the extracts of thrombocytopenic purpura as compared with the normal and control rabbits.

The only deviation from the constant findings was seen in the case of E. 1. In this case a reduced number of megakaryocytes was found. The megakaryocytes showed no outstanding changes in the cells, such as degeneration, pyknotic nuclei, or vacuolation of the cytoplasm. Sections of the spleen, liver, and lung were studied and failed to show any significant pathologic changes. Particular attention was directed to the examination of the lung sections to determine the presence of megakaryocytes, as reported by Howell and Donahue.<sup>4</sup>

#### DISCUSSION

It is well established that some relationship exists between the spleen and blood platelets. This is evidenced by the marked and rapid rise in the platelet

count following splenectomy on patients with essential thrombocytopenic purpura, a finding substantiated in all of our cases. The number of circulating blood platelets in the peripheral blood is dependent upon a normal maturation process in the bone marrow. Wright's<sup>17</sup> original finding that the megakaryocytes are the only source of true platelets has been repeatedly confirmed by many investigators. Frank,<sup>2</sup> Limarzi and Schleicher,<sup>6</sup> and Seeliger,<sup>11</sup> have noted in essential thrombocytopenic purpura an increase in the megakaryocytes of the bone marrow associated at times with immature forms. This suggests a maturation arrest of the megakaryocytic tissue and thus speaks for a lack of platelet formation rather than an increased destruction of these elements.

TABLE II  
MEGAKARYOCYTE COUNTS

| SPLenic EXTRACTS                          | BONE MARROW IMPRINTS | BONE MARROW SECTIONS |
|---|----------------------|----------------------|
| E. 1                                      | 8.1                  | 8                    |
| E. 2                                      | 14.3                 | 9                    |
| E. 3                                      | 14.7                 | 12                   |
| E. 4                                      | 13.8                 | 10                   |
| E. 5                                      | 11.2                 | 9                    |
| Control                                   | 11.7                 | 11                   |
| (Splenic vein thrombosis)                 |                      |                      |
| Bone marrow of a normal sacrificed rabbit | 12.5                 | 10                   |

Results described elsewhere in this report are not fully in accord with those of Troland and Lee.<sup>15, 16</sup> These workers report a depression of the platelet count to the level commonly seen in persons with essential thrombocytopenic purpura. For the most part, the preparation of the splenic extracts, weight of the splenic tissue, and the method of counting platelets in this study was similar to that employed by Troland and Lee.

It has been demonstrated that the five extracts prepared from the patients with essential thrombocytopenic purpura, when injected intravenously, produce a moderate depression of the circulating blood platelets. This reduction ranges from 70,000 to 177,000 blood platelets, and is very transient in duration. In practically every instance platelet counts returned to the preinjection level within twenty-four hours. Torrioli and Pusie<sup>14</sup> showed that large doses of an aqueous extract of a normal spleen caused a considerable reduction in the circulating blood platelets of rabbits when injected intravenously. An acetone extract of a normal spleen in this series produced no significant depression in the blood platelet count.

Because of the megakaryocytic hyperplasia found in essential thrombocytopenic purpura, bone marrow sections and imprints were obtained at the point of maximal depression of the blood platelets to determine the relationship of the thrombopenia to the megakaryocytes. No disturbances in the number or cell structure of the megakaryocytes were detected.

Due to the short-lived depression of the circulating blood platelets one may speculate that the extract contains a substance which interferes with the rate of formation of the platelets from the megakaryocytes. It is, of course, possible that if these injections were continued for some time an effect on the megakaryocytes would result.

## CONCLUSIONS

1. The intravenous injection of acetone splenic extracts of cases of essential thrombocytopenic purpura produces a transient significant depression of the platelet count in rabbits.

2. The splenic extracts exerted no effect upon the number or cell structure of the megakaryocytes of the bone marrow.

3. Specimens of the spleen, liver, and lungs of rabbits obtained after the injection of thrombocytopenic splenic extracts showed no distinctive pathologic changes.

4. The intravenous injection of acetone splenic extracts from six control cases produced no significant change in the level of the platelet count in rabbits.

I wish to express my gratitude to Dr. Robert W. Keeton and Dr. Louis R. Limarzi for their invaluable advice and criticism.

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## THE RELIEF OF MUSCULAR WEAKNESS BY PYRIDOXINE HYDROCHLORIDE\*

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THE fraction of the vitamin B complex known as B<sub>6</sub> has recently been synthesized. It is chemically described as 2-methyl-3-hydroxy-4, 5-di-(hydroxymethyl)-pyridine.<sup>1, 2</sup> The Council on Pharmacy and Chemistry of the American Medical Association has accepted pyridoxine as the official name of this vitamin. The hydrochloride is to be known as pyridoxine hydrochloride.<sup>3</sup> A deficiency of this substance in rats results in impaired growth and a characteristic dermatitis.<sup>4</sup> Little is known concerning its physiologic activity, but there is some evidence that it is concerned with storage of fat<sup>5</sup> and with the utilization of unsaturated fatty acids.<sup>6</sup>

Our interest was aroused by the early report of Spies and others<sup>7</sup> on the spectacular influence of pyridoxine in relieving the muscular weakness of pellagrins. He reported that pellagrins who had been relieved as regards most signs and symptoms of the disease by vigorous treatment with nicotinic acid, thiamin chloride, and riboflavin, nevertheless still manifested extreme muscular weakness and fatigue. At this time the intravenous administration of 50 mg. of pyridoxine enabled patients (who still complained of extreme nervousness, insomnia, irritability, abdominal pain, weakness, and difficulty in walking) to experience dramatic relief of all these symptoms within as short a time as four hours. One of these patients, who had been unable to walk more than a few steps, walked two miles within twenty-four hours after the injection of 50 mg. of pyridoxine.

In view of Spies' results it seemed worth while to test the action of pyridoxine in patients suffering from clinical conditions characterized by extreme muscular weakness and exhaustion, in which no other signs or symptoms of a vitamin B complex deficiency were evident.

### METHODS

Because of the difficulty of quantitating the patient's subjective feelings of muscular weakness, our tests were based on the ability of the individual to perform work to the point of exhaustion under standard conditions. For this purpose we employed two procedures:

(1) *Leg Test*.—With the patient in the supine position in bed, he was required to lift the extended right leg to the vertical position, at the rate of 24 times per minute, for as long as he was able to continue.

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(2) *Arm Test*.—In this test the work was done with the right hand and arm. While sitting up in bed, the patient was required to raise a 10-pound weight a distance of 13 inches, at a rate of 30 times per minute. He grasped a comfortable handle attached to a rope which ran over a pulley fixed at the foot of the bed. The resting position for this exercise was with the upper arm approximated to the lateral thoracic wall, so that the essential movement was a flexion of the forearm on the upper arm from the initial 90-degree angle to about a 30-degree angle.

The arm and leg tests were performed twice a day, with an intervening rest period of fifteen minutes. They were always conducted by the same observer in the same environment and at the same time of day. The tests were not considered satisfactory unless the results of two tests on the same limb coincided fairly closely. The average of these two tests was considered to be the work performance of the limb for the day. Preliminary experiments in normal people showed that the identical muscle testing procedures, performed daily, gave no indication of any increase in muscular ability due to training.

In order to avoid the possible psychologic effects of the therapy, all patients were given intravenous injections of saline daily from the beginning. When a constant base line of muscular ability had been established for a particular patient, a solution of pyridoxine hydrochloride\* was substituted for the saline without the patient's knowledge. In some cases, after the results of the vitamin had been observed, the saline was again resumed as a further check upon the enthusiasm aroused in the patient by the beneficial effect of the treatment.

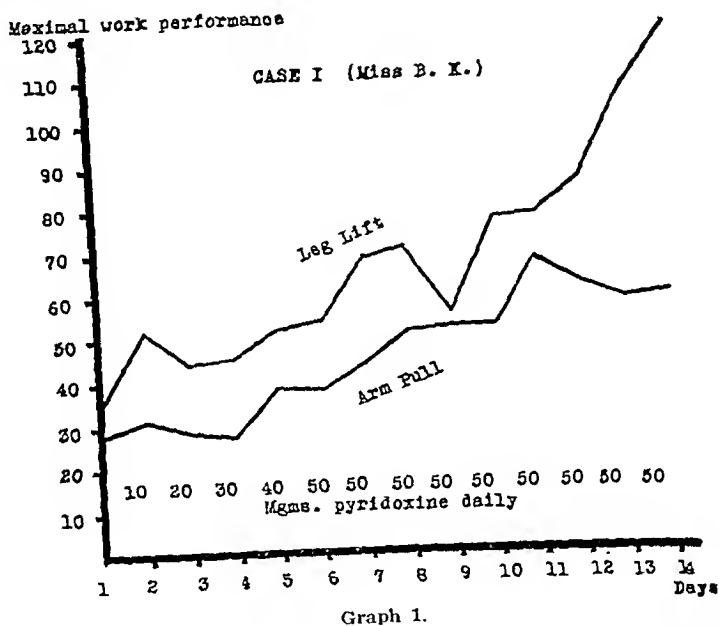
In our earliest cases varying doses of pyridoxine hydrochloride from 10 mg. to 50 mg. intravenously per day were used. We subsequently adopted a standard dose of 50 mg. a day for all cases for the purposes of this work. No toxic or ill effects of any kind were ever observed to occur from the use of these amounts of the vitamin.

The following is a summary of our results. Brief case histories are appended at the conclusion of this article.

*Neurasthenia*.—Our first case (Miss B. K.) was a 27-year-old female, whose ailment had never been definitely diagnosed. Physical and laboratory examinations had been consistently negative in the hands of numerous physicians, and at no time were any clinical signs of vitamin deficiency noted. She complained primarily of weakness and fatigue to such an extent that she had been practically bedridden for eight years. She was barely able to make the daily trip to the hospital for examination and treatment. We started this patient on pyridoxine hydrochloride without any preliminary tests with saline. Within five days she began to show increased muscular ability, as measured by our objective tests (Graph 1). Her appetite improved markedly and her weight rose from 99 to 104 pounds. After two weeks of treatment the patient stated that she was able to be up and out of bed for four hours a day. At this point we began to inject saline intravenously in place of the pyridoxine hydrochloride. Our objective tests showed no decrease in the patient's muscular strength, but she insisted that her weakness was returning and that she was no longer able to be

\*Supplied by Merck & Co., through the courtesy of Dr. D. F. Robertson.

out of bed long enough to report for daily intravenous injection. We gave her 50 mg. tablets of pyridoxine hydrochloride to be taken orally, once a day at home, and advised her to return after two weeks. When next seen the patient reported that her strength was again returning. We now resumed the daily intravenous injections of pyridoxine hydrochloride. After fourteen more injections the patient was able to be up and about eight hours a day. We discontinued treatment and advised her to take fresh yeast at home. She has continued to improve and when last seen, five months after beginning pyridoxine, she was able to be out of bed eight hours daily, was attending a few social functions, and her weight had increased by 25 pounds.



Graph 1.

*Hyperthyroidism.*—We attempted the use of pyridoxine hydrochloride in three patients with hyperthyroidism, each of whom complained of marked weakness and fatigability. In every case there was a definite increase in muscular ability (Graph 2 illustrates one such case). Lugol's solution was withheld until we were able to demonstrate the effect of the vitamin. During this period the basal metabolic rates which were determined showed little change, so that the increase in strength could not be attributed to changes in the basal metabolic rate. In one case the pyridoxine hydrochloride was discontinued as soon as the patient showed increased strength and lugolization was begun. There was no further gain in strength. The Lugol's solution was continued and whole yeast was added to the diet. He immediately began to show further improvement as concerns muscular ability, although during this entire period his basal metabolic rate showed little variation.

*Ulcerative Colitis.*—Decided improvement in muscular ability was obtained with pyridoxine hydrochloride in 4 patients with ulcerative colitis. Objective tests could be made in only 2 of these patients, the others being unable to under-



days of pyridoxine hydrochloride administration, she was able to sit up in bed without support and to feed herself. After fourteen days of treatment she was able to begin walking. In no case were we able to note any improvement in the ulcerative colitis per se as regards frequency and number of stools.

*Myasthenia Gravis.*—Pyridoxine hydrochloride was of no apparent benefit in one case of myasthenia gravis which had been previously well controlled with prostigmin. For the purposes of observation, prostigmin was discontinued the previous night, and the patient was given 50 mg. of pyridoxine hydrochloride intravenously in the morning. By 6:00 P.M. the patient began to show acute signs of muscular weakness, as evidenced by her inability to eat or even swallow water. She was now given 100 mg. of pyridoxine hydrochloride intravenously, but showed no response. One-half hour later prostigmin was given, to which she responded almost immediately.

*Malnutrition.*—In 2 patients with anorexia nervosa and in one patient with cardiospasm we obtained no evidence of increased strength following the use of pyridoxine hydrochloride. All 3 patients were markedly underweight, were exceedingly weak, subsisted on deficient diets, and showed clinical signs of vitamin deficiencies. None of these patients showed any real response after receiving eight to fourteen daily injections of pyridoxine hydrochloride.

#### SUMMARY AND DISCUSSION

Noteworthy relief of muscular weakness has been obtained following the intravenous administration of pyridoxine hydrochloride in one patient with so-called neurasthenia, 3 patients with hyperthyroidism, and 4 patients with ulcerative colitis. No results were obtained in one patient with myasthenia gravis and in 3 patients with severe malnutrition. It is evident that our good results were obtained in patients in whom one might expect to find either a deficiency of vitamin intake or a vitamin requirement in excess of the normal intake, even though no recognizable symptoms of vitamin deficiency other than weakness were present. However, we cannot explain why the 3 patients with severe malnutrition with obvious manifestations of multiple vitamin deficiencies did not respond to pyridoxine hydrochloride. It is suggested that these failures might have been due to the absence of other vitamin factors which are essential to the action of pyridoxine hydrochloride.

#### CASE REPORTS

CASE 1 (Miss B. K.).—The essential details of the history and the results of treatment (Graph 1) with pyridoxine hydrochloride have been summarized above. It is worthy of note that this patient had been on a subminimal diet for eight years, eating from 800 to 1,000 calories a day, with very little meat and whole grain foods.

Physical examination showed a tall, thin female, 5 feet 9 inches in height, and weighing only 101 pounds. There were no other positive physical signs. Bronchoscopy showed an atrophic and hyperemic mucosa.

Laboratory Studies: Hemoglobin 80 per cent; erythrocytes 4,400,000; leucocytes 7,750 with a normal differential; urine negative; sugar 64 mg. per cent; nonprotein nitrogen 27 mg. per cent; chlorides 494 mg. per cent; cholesterol 298 mg. per cent; icterus index 6; carbon dioxide combining power 57.3 per cent; van den Bergh, direct and indirect, both negative; sputum negative for tubercle bacilli.

CASE 2 (Mrs. L. K.).—A white, divorced, 24-year-old female, entered the hospital with nervousness, weight loss, weakness, and prominence of the eyes for the past year.

Physical examination showed a marked exophthalmos, a diffusely enlarged thyroid gland, moist warm skin, a fine tremor of the hands, rapid pulse (90 to 110), and a blood pressure of systolic 130 and diastolic 70.

Laboratory studies were essentially negative, except for a basal metabolic rate of +41.

The report of the pathologist on the appearance of the thyroid after removal was consistent with the diagnosis of diffuse toxic goiter.

This patient received no Lugol's solution during the period of administration of pyridoxine hydrochloride, and her basal metabolic rate showed no noteworthy change. Nevertheless her muscular ability increased markedly, as shown in Graph 2.

At this time the patient refused to stay in the hospital because of difficulties at home. She received no medication while at home, and returned to the hospital one week later with the same signs and symptoms. Her basal metabolic rate was still +41. Lugol's solution (ten drops t.i.d.) was begun, without any pyridoxine hydrochloride. The patient showed no further increase in muscular ability, although her basal metabolic rate dropped to +13.

CASE 3 (Mr. S. D.).—A white, married, 35-year-old male, entered the hospital with nervousness, feeling of warmth and perspiration, palpitation, polyuria, diarrhea, enlargement of the neck, and the loss of 30 pounds of weight within two months.

Physical examination showed a slight exophthalmos, a diffusely enlarged thyroid, warm, moist skin, a fine rapid tremor of the hands, a pulse rate of 100, and a blood pressure of systolic 144 and diastolic 72.

The only significant laboratory test was a basal metabolic rate of +80.

The postoperative examination of the gland showed a diffuse toxic goiter, consistent with the clinical diagnosis of diffuse toxic goiter with hyperthyroidism.

This patient showed an increase in muscular ability immediately after his first dose of pyridoxine hydrochloride. After he had received five daily doses it was necessary to stop the pyridoxine and to start Lugol's solution because of his continued toxic state. However, by this time his arm pull had increased from 35 to 44, and his leg lift from 45 to 85. The basal metabolic rate taken on admission was +81, and after pyridoxine hydrochloride it was +72.

Five days after the beginning of treatment with Lugol's solution (10 drops t.i.d.) the basal metabolic rate dropped to +53, but there was no change in muscular ability. Whole yeast was then added to the diet, and within seven days the arm pull increased to 38 and the leg lift to 260. The basal metabolic rate at this time was +50.

CASE 4 (Mrs. B. R.).—A white, married, 38-year-old female, entered the hospital with nervousness, palpitation, a swelling in the neck, and weight loss over a period of three months.

There was a marked exophthalmos, a diffusely enlarged thyroid, a fine rapid tremor of the hands, warm moist skin, rapid pulse rate (105), and a blood pressure of systolic 140 and diastolic 80.

Repeated basal metabolic rates ranged from +80 to +40. Other laboratory studies were negative.

The diagnosis of diffuse toxic goiter with hyperthyroidism was confirmed by examination of the thyroid postoperatively.

This patient was given thiamin chloride, 15 mg. intravenously, for five days, but showed no increase in strength. Pyridoxine hydrochloride was then substituted for the thiamin chloride, and within ten days her leg lift increased from 40 to 250, and her arm pull from 16 to 85. Her basal metabolic rate remained unchanged during this period.

CASE 5 (Mr. L. L.).—A white, 19-year-old schoolboy, with a history of chronic non-specific ulcerative colitis of three years' duration, entered the hospital complaining of an acute exacerbation of the colitis.

Physical examination disclosed an emaciated youth, with a right lower abdominal scar, the site of an appendectomy three years previously. Proctoscopic examination showed a hyperemic mucous membrane. Radiographic studies were consistent with a diagnosis of chronic ulcerative colitis.

No causative specific organisms could be found on repeated stool examinations. Hemoglobin was 50 per cent and erythrocytes were 2,890,000.

Medical treatment was attempted, consisting, over a period of several weeks, of a course of neoprontosil, repeated transfusions, iron, high vitamin high-caloric diet, and autogenous vaccine. The patient failed to show any improvement. An ileostomy was performed six weeks after admission.

We first saw the patient three months after admission. He was weak and emaciated; he had developed contractures of both legs and was having frequent, severe rectal hemorrhages.

Because of the state of his leg muscles it was possible to test only his arms. These showed the definite improvement in muscular ability summarized in Graph 3. This increase was paralleled by a general feeling of increased strength. At this time a hemicolectomy was performed and the patient died soon afterwards. Post-mortem examination showed a non-specific colitis complicated by a diffuse peritonitis.

CASE 6 (Mr. W. N.).—A white, 26-year-old male, German refugee, had an eleven-year history of chronic ulcerative colitis. Seven years prior to his admission he had had an ileostomy performed in Germany with but little relief; he continued to have acute exacerbations of his colitis. When we first saw him, he had entered the hospital for the second time within a month. He was having five to six bloody mucous stools a day. He had developed a crop of furuncles over his legs and arms, and he had swollen, tender, reddened wrists and knee joints. He was extremely emaciated and so weak that he could not sit up in bed. Laboratory studies were negative. It was impossible to perform any objective tests because of the arthritis and marked weakness. He had been receiving thiamin chloride intravenously (15 mg. daily), to which we added pyridoxine hydrochloride (50 mg. daily). The next day he was sitting up in bed, asking to be allowed up, and his appetite was excellent. We continued to give the pyridoxine hydrochloride for one week. The patient continued to evidence increased strength, but neither his colitis per se nor his arthritis changed. Pyridoxine hydrochloride was discontinued after a week and fresh yeast (three cakes a day) was added to his diet. He was discharged two weeks later, his arthritic symptoms were gone, and his colitis improved. He was advised to continue taking yeast at home.

He re-entered the hospital six weeks later with a re-exacerbation of his colitis and arthritis. He had not taken any yeast because of the "gaseous feeling" which he claimed followed its use. On this admission the patient failed to show any further response to pyridoxine hydrochloride.

CASE 7 (Mrs. J. L.).—A 54-year-old white, married female entered the hospital with cramping abdominal pains and a diarrhea of two weeks' duration.

Physical examination was negative except for a rapid irregular pulse rate. Radiographic, proctoscopic, and laboratory studies established the diagnosis of nonspecific ulcerative colitis complicated by a chronic cystitis and an auricular fibrillation of undetermined etiology. The patient was treated symptomatically with sedatives, transfusions, and high caloric low-residue diet. On an empirical basis she was given a treatment with carbarsone and emetine hydrochloride, and later sulfanilamide therapy. To supplement her diet she was given thiamin chloride, 25 mg. intravenously, nicotinic acid 25 mg., b.i.d., cevitamic acid 200 mg., b.i.d., and liver extract 1 c.c. intramuscularly. In spite of these measures her condition remained unchanged.

When first seen by us, the patient had been in the hospital for eight weeks. Because of her cardiac condition and marked weakness, objective tests were impossible. Her weakness had progressed so far that she even required help to eat. We gave her intravenous saline for five days with no effects. We then started pyridoxine hydrochloride. The next day the patient felt much stronger, responded readily to questions, and noted an improvement in her appetite. By the time 150 mg. had been given (three daily doses) she was able to sit up in bed for five minutes without assistance. After ten doses she was sitting up in a wheel chair, and after two weeks of treatment she began to take a few steps; she was then discharged from the hospital. At no time during this period was there any change in frequency and number of stools.

CASE 8 (Mr. E. S.).—A white, married, 40-year-old male, when first seen had weakness and fatigue. Three months ago he had had a colostomy performed because of chronic ulcerative colitis. His colitis had improved, but he still complained of fatigue on slight exertion.

Physical examination was negative, except for the colostomy opening. Laboratory studies offered no aid.

This patient was started on pyridoxine hydrochloride and showed a gradual improvement in strength. At the end of ten days his arm pull had increased from 35 to 70 and his leg lift from 30 to 90. During two weeks of treatment he gained 10 pounds; in the previous three weeks he had gained only 3 pounds.

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### COMPARATIVE EFFECTS OF PROPYLENE GLYCOL AND SOME OTHER AGENTS ON OXYGEN CONSUMPTION OF THE ORGANISM\*

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THE voluntary running activity of white rats receiving propylene glycol was found previously to be increased somewhat.<sup>1</sup> These rats were not depressed, and they stored more glycogen than normal animals. The oxygen consumption of isolated liver perfused with propylene glycol was decreased, and the organ stored glycogen simultaneously.<sup>2</sup> These results are related to the possible food value of propylene glycol, which has a definite glycogenic action. But some increase in general activity should theoretically increase oxygen consumption or leave it unchanged. Therefore, it was thought desirable to investigate further the metabolic actions of this glycol, using oxygen consumption as the criterion. This report presents the results obtained.

#### METHOD

The apparatus used was of the closed-circuit type, a unit consisting of a Hempel desiccator acting as a rat chamber, which was connected by glass tubing and a three-way stopcock to a large glass cylinder of 4,500 c.c. capacity and

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graduated at 10 c.c. intervals. Soda lime and calcium chloride were put in the desiccator to absorb carbon dioxide and water. The large cylinder was filled with pure oxygen. As a rat used oxygen, water was allowed to siphon into the large cylinder from an 18-liter reservoir, thus equalizing the pressure inside the system with that of the atmosphere. The amount of water siphoned over was a measure of the oxygen consumed, after appropriate corrections for changes in temperature and barometric pressure. Six such units were operated at once.

The rats were observed for seven hours in this metabolic arrangement. No rat was used for more than four consecutive days. Usually 2 runs with the use of glycol or other agents were made, alternated with 2 control runs. The oxygen consumption was expressed as cubic centimeters of oxygen for 100 sq. cm. of body surface per minute. Since the metabolism varied with the environmental temperature, the control observations on each rat were plotted against the environmental temperature and the line through these points calculated by the method of least squares. The point at which this line crossed the 22°-axis was chosen to represent the average control result. Direct comparison of the results on controls and with different agents was made by moving the experimental points parallel to the control line until the 22°-axis was reached. The values so obtained were averaged, and the averages are summarized in Table I.

The nutritional state of the animals and the environmental temperature were as close to those of previous feeding and activity experiments as possible.<sup>1</sup> The animals were fed the usual stock diet of this department and were not fasted prior to the determination of oxygen consumption. The environmental temperature was not held constant but was carefully observed, and the mean values of oxygen consumption were corrected for the variations in temperature.

The doses of propylene glycol and other agents used and methods of administration were as follows: propylene glycol, 5 c.c. and 10 c.c. per kilogram, gastrically; benzedrine, 2 mg. per kilogram, subcutaneously; pentobarbital, 30 mg. per kilogram, subcutaneously; and chloral hydrate, 250 mg. per kilogram, intraperitoneally. The other agents, besides glycol, were used as a further check on the results with the glycol. Benzedrine, which is a general excitant, was expected to increase the oxygen consumption, and pentobarbital and chloral, depressants, were expected to decrease it. However, only the benzedrine acted according to expectations. A total of 505 determinations on 19 rats was made. The duration of each observation was seven hours. The other agents were compared in the same rats receiving the glycol.

#### CONTROL RESULTS

A total of 256 control observations on 19 rats showed an average oxygen consumption of 1.363 c.c. per 100 sq. cm. per minute at 22° C. (Table I). This value had a variability of less than 3 per cent.

#### GLYCOL AND OTHER AGENTS

Propylene glycol, in both doses used, depressed the oxygen consumption significantly, i.e., down to an average of 1.142 c.c. from the control average of 1.363 c.c. Benzedrine raised the oxygen consumption in 14 rats and increased

TABLE I  
OXYGEN CONSUMPTION OF RATS RECEIVING PROPYLENE GLYCOL AND OTHER AGENTS

| RAT | CONTROL            |                  |        | PROPYLENE GLYCOL<br>10 C.C. PER KG. |                  |                    |                  | BENZEBINE          |                  | PENTOBARBITAL      |                  | CHLORAL            |                  |
|-----|--------------------|------------------|--------|-------------------------------------|------------------|--------------------|------------------|--------------------|------------------|--------------------|------------------|--------------------|------------------|
|     | DETERM-<br>NATIONS | O <sub>2</sub> * | S.E.†  | DETERM-<br>NATIONS                  | O <sub>2</sub> * | DETERM-<br>NATIONS | O <sub>2</sub> * | DETERM-<br>NATIONS | O <sub>2</sub> * | DETERM-<br>NATIONS | O <sub>2</sub> * | DETERM-<br>NATIONS | O <sub>2</sub> * |
| 1   | 8                  | 1.502            | ±0.009 | 4                                   | 1.138            |                    |                  | 4                  | 1.974            |                    |                  |                    |                  |
| 2   | 9                  | 1.285            | ±0.016 | 4                                   | 1.147            |                    |                  | 4                  | 1.593            |                    |                  |                    |                  |
| 3   | 7                  | 1.431            | ±0.032 | 3                                   | 1.303            |                    |                  | 3                  | 1.735            |                    |                  |                    |                  |
| 4   | 9                  | 1.327            | ±0.029 | 5                                   | 1.158            |                    |                  | 3                  | 1.668            |                    |                  |                    |                  |
| 5   | 22                 | 1.315            | ±0.026 | 5                                   | 1.269            | 5                  | 1.132            | 4                  | 1.585            | 5                  | 1.273            |                    |                  |
| 6   | 6                  | 1.324            | ±0.047 | 3                                   | 1.229            |                    |                  | 3                  | 1.661            |                    |                  |                    |                  |
| 7   | 18                 | 1.249            | ±0.030 | 5                                   | 1.241            | 5                  | 1.470            | 5                  | 1.476            | 5                  | 1.485            |                    |                  |
| 9   | 19                 | 1.275            | ±0.034 | 5                                   | 1.112            | 4                  | 1.309            | 5                  | 1.597            | 4                  | 1.163            |                    |                  |
| 10  | 19                 | 1.252            | ±0.035 | 5                                   | 1.017            | 5                  | 1.183            | 5                  | 1.448            | 5                  | 1.263            |                    |                  |
| 11  | 15                 | 1.300            | ±0.036 | 5                                   | 1.103            | 2                  | 1.071            | 5                  | 1.525            | 2                  | 1.220            |                    |                  |
| 12  | 19                 | 1.457            | ±0.039 | 5                                   | 1.065            | 5                  | 1.309            | 5                  | 1.670            | 5                  | 1.343            |                    |                  |
| 14  | 20                 | 1.348            | ±0.029 | 5                                   | 1.205            | 5                  | 1.329            | 5                  | 1.629            | 5                  | 1.243            |                    |                  |
| 15  | 21                 | 1.426            | ±0.041 |                                     |                  | 5                  | 1.437            |                    |                  | 5                  | 1.152            | 5                  | 1.363            |
| 16  | 6                  | 1.290            | ±0.086 | 4                                   | 0.917            |                    |                  | 2                  | 1.377            |                    |                  |                    |                  |
| 17  | 7                  | 1.435            | ±0.038 | 4                                   | 1.099            |                    |                  | 3                  | 1.560            |                    |                  |                    |                  |
| 18  | 19                 | 1.452            | ±0.036 |                                     |                  | 5                  | 1.355            |                    |                  | 5                  | 1.475            |                    |                  |
| 20  | 11                 | 1.242            | ±0.029 |                                     |                  | 5                  | 1.135            |                    |                  | 5                  | 1.074            | 5                  | 1.400            |
| 21  | 11                 | 1.352            | ±0.045 |                                     |                  | 5                  | 1.325            |                    |                  | 4                  | 1.629            |                    |                  |
| 22  | 12                 | 1.650            | ±0.068 |                                     |                  | 5                  | 1.531            |                    |                  | 5                  | 1.519            |                    |                  |
| 258 |                    | 1.363            |        | 62                                  | 1.142            | 56                 | 1.289            | 56                 | 1.607            | 55                 | 1.319            | 10                 | 1.381            |

\*Oxygen consumption = average c.c. oxygen consumed per 100 sq. cm. of body surface per minute at 22° C.  
†Standard error of mean.

the activity, i.e., increased general excitability but did not cause tremors; the oxygen consumption was raised to 1.607 c.c. Interestingly enough, pentobarbital decreased the oxygen consumption only slightly in 12 rats. This was interesting, since the animals which received propylene glycol behaved in the same way as did the unmedicated controls and were not narcotized, whereas those receiving pentobarbital were markedly depressed and, in fact, comatose, although the reflexes could be elicited. Recovery occurred at the end of a seven-hour period. Therefore, the depression of oxygen consumption caused by propylene glycol was not due to a decrease in general activity. This confirmed the observations on the isolated perfused liver and explained why the rats, although showing no depression of running activity, were able to store energy (liver glycogen) better than control rats. The burning of propylene glycol requires less oxygen to release a given amount of energy than do most other foodstuffs (except fat).

The results in 2 rats with chloral, which markedly depressed these animals and caused coma with loss of reflexes, were also interesting in that the oxygen consumption was practically the same as that of the controls, contrary to expectations. However, the results with the agents, other than glycol, are of value in showing that the depressant effects of propylene glycol on oxygen consumption are peculiar to this agent, since all other agents tried, except benzedrine, compared favorably with the controls. The explanation of this effect of propylene glycol is not at hand, although the results in the whole animal are consistent with a similar depression of oxygen consumption of the isolated liver perfused with this glycol. This storage of glycogen in the isolated liver, as well as in whole animals, indicates storage of energy for which there is less need of oxygen, despite a fair general activity of the animals.

#### CONCLUSIONS

1. Propylene glycol was found to depress the oxygen consumption of white rats, this depression not being due to a decrease in activity, since the depressions of pentobarbital and chloral in the same rats did not significantly decrease the oxygen consumption.

2. These results on whole animals are in agreement with previous results on isolated liver perfused with propylene glycol, which also showed a decrease in oxygen consumption.

3. The doses of propylene glycol used were beyond those likely to be taken as solvent or vehicle in food or medicinal agents, but the depression of oxygen consumption could occur with very large or toxic doses. This result is essentially of scientific importance, because the use of propylene glycol has a wide margin of safety.

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# CLINICAL CHEMISTRY

## INSULIN FERRIHEMOCHROMOGEN\*

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DURING the course of an investigation on the conjugation of ferriheme (hematin) with various nitrogenous substances in liquid ammonia (a process by which it is possible to prepare ferrihemochromogens which are soluble in aqueous media at physiologic pH) it was desired to study the reactions of ferriheme with a protein in this solvent. Insulin was used in this particular instance because it is one of the few proteins which is soluble in anhydrous ammonia.<sup>1, 2</sup> When it was discovered that certain insulin ferrihemochromogens formed by this reaction showed a modification of the characteristic physiologic activity of the hormone, this modification was studied. The results indicate for these soluble insulin conjugates some of the properties possessed by the protamine conjugate and other relatively insoluble depot forms of insulin.

Certain nitrogenous substances combine with ferriheme to give rise to a class of compounds called ferrihemochromogens. Among these nitrogenous substances termed "suitable" by Bertin-Sans and de Montessier, are ethylamine, ammonia, aniline, glycine and taurine,<sup>3</sup> nitrite<sup>4</sup> (in the nitro form), cetyl pyridinium chloride and nicotinamide<sup>5</sup> and cyanide (in the isonitrile form). Some nitrogenous substances are "unsuitable" in this sense; acetanilid and sulfanilamide<sup>6</sup> and triethanolamine<sup>6</sup> come into the latter category. The presence of quaternary nitrogen seems to be essential to the formation of a coordination compound with the iron of ferriheme.<sup>†</sup>

Basic proteins, which contain a large proportion of quaternary nitrogen, can combine with large amounts of ferriheme. Globin can, in fact, take up fully thirty times the quantity of heme as there exists, preformed, in hemoglobin. This capacity of globin is exceeded by that of insulin which was found to be capable of combination with a maximum of about eight times its weight of ferriheme chloride—a ratio of one iron atom to each nitrogen of the insulin molecule.

### EXPERIMENTAL DATA

Crystalline insulin assaying 24 units per milligram or amorphous insulin assaying 20 units per milligram was dissolved in anhydrous ammonia in a

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†Another type that with hydro-acid radicals of small ionic volume, is possible. polar in nature and have been termed "hemochromogens." The term "hemochromogen" is more appropriate. "Suitable" ferriheme formers may or may not contain nitrogen, and this need not be in the quaternary form, viz.; azide ion,<sup>3</sup> cyanide ion (in the nitrile form<sup>9</sup>) and cyanamide ion.<sup>3</sup> The combinations of insulin with ferriheme appear to be exclusively of the ferrihemochromogen type.

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pyrex Dewar flask and treated with various amounts of ferriheme chloride.<sup>10</sup> The ammonia was allowed to boil off at room temperature through a mercury trap, the last traces being removed by a suction pump. The insulin ferriheme conjugate was less soluble in anhydrous ammonia than either of its components, for, before all the ammonia boiled off, it precipitated as a gelatinous strawberry-colored mass. This became a greenish-black residue on removal of all the ammonia. This residue was dissolved in water to a final concentration of 10 units per cubic centimeter calculated on the basis of original insulin content.

TABLE I

| WEIGHT OF RABBIT (KG.) | MATERIAL      |                            |       | TIME INTERVAL |      | BLOOD SUGAR (MG. PER 100 C.C.) | REMARKS                                |
|------------------------|---------------|----------------------------|-------|---------------|------|--------------------------------|--|
|                        | INSULIN UNITS | INSULIN FERRIHEMOCHROMOGEN |       |               |      |                                |  |
|                        |               | RATIO                      | UNITS | HR.           | MIN. |                                |  |
| 2.88                   | 10            |                            |       | 0             |      | 97                             | Intravenously                          |
|                        |               |                            |       | 1             | 41   | 52                             |  |
|                        |               |                            |       | 24            | 36   | 102                            | 1 hour postprandial                    |
| 3.05                   |               | 1:6                        | 15    | 0             |      | 99                             | Intraperitoneally                      |
|                        |               |                            |       | 1             | 21   | 81                             |  |
|                        |               |                            |       | 24            | 26   | 81                             | 1 hour postprandial                    |
| 3.58                   |               | 1:6                        | 20    | 0             |      | 113                            | Intravenously                          |
|                        |               |                            |       | 1             | 29   | 56                             |  |
|                        |               |                            |       | 24            | 40   | 77                             | 1 hour postprandial                    |
| 1.22                   | 5             | 1:8                        | 5     | 0             |      | 108                            | Intraperitoneally                      |
|                        |               |                            |       | 1             | 30   | 53                             |  |
|                        |               |                            |       | 3             | 40   | <45                            | Convulsions (20 c.c. 10% dextrose)     |
|                        |               |                            |       | 22            | 40   | 75                             | Intraperitoneally                      |
|                        |               |                            |       | 23            | 10   | <45                            | Convulsions                            |
|                        |               |                            |       |               |      |                                |  |
| 1.76                   |               | 1:8                        | 10    | 0             |      | 106                            | Intraperitoneally                      |
|                        |               |                            |       | 1             | 30   | 59                             |  |
|                        |               |                            |       | 3             | 20   | <45                            | Convulsions (20 c.c. 10% dextrose)     |
|                        |               |                            |       | 22            | 40   | 56                             | Post-mortem sample Found dead in rigor |

A few of the theoretically numberless pigment conjugates with varying insulin and ferriheme ratios were prepared. One with a high ratio (5:1 of insulin to ferriheme), one with a low ratio (1:6), and one which was completely saturated with ferriheme (1:8 insulin ferrihemochromogen) were tested for physiologic activity. The first showed no difference in activity from that exhibited by ordinary insulin when given in the same manner and dosage. Subcutaneous injection postprandially of 10 units in a 2.72 kg. rabbit caused convulsions in 208 minutes, while the control solution of ordinary insulin under the same conditions brought the blood sugar level to the convulsive level in a litter mate in 197 minutes. The effect of insulin-ferrihemochromogen ratios of 1:6 and 1:8 on the blood sugars of rabbits is given in Table I. There appears to be a definite prolongation of the insulin effect even when these conjugates are given intravenously or intraperitoneally, a condition which does not obtain when the depot forms of insulin are given in this manner.<sup>11</sup> The 1:8 ratio of insulin and ferrihemochromogen (which is fully saturated with the pigment

as evidenced by the settling from solution of small amounts of ferriheme) was also tested by the mouse method. Ten animals were convulsed in an average of seventy minutes while one-fourth the dosage of ordinary insulin caused convulsions in 10 mice in an average time of twenty minutes.

No toxic manifestations other than for the insulin effect have been noted in any of the animals used, some of which have been pushed to the convulsive level repeatedly by the administration of the insulin ferriheme conjugate over the past year.

The nitric oxide and carbon monoxide derivatives of 1:8 insulin ferrihemochromogen have been prepared. Their study is in progress and is of interest since they appear to be somewhat resistant to tryptic digestion. Attempts to form the cyanamide and the cyanide derivatives of 1:8 insulin ferrihemochromogen have not been successful.

#### SUMMARY

A series of water-soluble conjugates formed by the reaction of insulin and ferriheme chloride in anhydrous ammonia have been prepared. Insulin combines with ferriheme to a maximum ratio of 1:8 where a relationship of one insulin nitrogen to one ferriheme iron atom exists.

Chemical studies indicate that the combination is similar to that formed by nicotinates and ferriheme where the nitrogen of the former is coordinated in the  $4p$  electronic orbit of the iron (ferrihemochromogen linkage) and is different from that formed between cyanamide and ferriheme where the nitrogen is polar covalent with the  $3p$  orbit (ferrihemide linkage).

The molecular weight of 1:8 insulin ferrihemochromogen is calculated to be in the neighborhood of 315,000. Consequently, it resembles in physiologic action the "depot" forms of insulin, and, even with intravenous or intraperitoneal injection, administration to rats and rabbits results in hypoglycemia which is delayed in its onset and prolonged in duration.

Repeated administrations in rabbits have indicated neither acute nor chronic toxic effects other than for the hypoglycemic convulsions induced, and this is considered as evidence against the reputed toxicity of ferriheme (hematin).

I wish to acknowledge my indebtedness to Dr. R. G. Roberts, of the Chicago Medical School, and to Dr. Walter S. Bridgins, of Armour and Company, for their material help in the investigation.

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## CLINICAL USE OF THE CEPHALIN-CHOLESTEROL FLOCCULATION TEST\*

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WE BECAME interested in the cephalin-cholesterol flocculation test, as developed by Hanger,<sup>1</sup> because of the simplicity of this method for evaluating "irritation of liver cells" and the significance of clinical jaundice. We have had the opportunity to study the test in 114 patients and present the results as indicating that the test can be of definite clinical value.

Recently Rosenberg<sup>2</sup> has reported a considerable series which confirms the statements of Hanger. In 155 selected cases, he found that the test was reliably correlated with the clinical findings and felt that negative or slight reactions are indicative of obstructive jaundice, while prompt and strong reactions are indicative of parenchymatous jaundice.

The test is performed by adding to 4 c.c. of normal saline solution 1 c.c. of cephalin-cholesterol suspension<sup>3</sup> and 0.2 c.c. of the serum to be tested. After mixing, the tube is allowed to stand at room temperature for twenty-four hours, then read. A homogeneously cloudy solution is read as a negative test. Complete flocculation and precipitation, with a clear supernatant fluid, is read as four-plus. We have considered three-plus and four-plus tests as "positive" and one-plus and two-plus tests as "doubtful."

For confirmation, as many tests as possible were performed on each patient. In most cases, the icterus index, total protein and partition, and total cholesterol were determined, and in many cases bromsulfalein or galactose tolerance tests were done as well. It was impossible to obtain pathologic or operative proof in all cases, but cases were excluded from consideration unless the diagnosis was reasonably established.

In Group I, there were 83 cases with no clinical or laboratory evidence of intrinsic liver disease or of biliary obstruction. Of these, only 4 showed positive tests. Two were in cases of hyperthyroidism, one coincident with severe pernicious anemia. One was in a case of Cooley's anemia with a markedly enlarged liver. The fourth was a case of bronchial asthma and probable periarteritis nodosa. In each of these it is conceivable that liver damage or irritation was present in the absence of other positive tests. It is well known that

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The cephalin-cholesterol mixture used in our tests was kindly furnished by Dr. F. M. Hanger. Method of preparation can be found in the literature.<sup>1,2</sup>

liver damage occurs in hyperthyroidism. There were 12 more cases with doubtful tests. Of these, 3 were cases of hyperthyroidism, and one was a case of primary disseminated arteritis. In some of these repeated tests were later negative.

In Group II there were 22 cases with evidence of intrinsic liver disease. The 4 cases of catarrhal jaundice were all positive and became negative with the subsidence of clinical jaundice. Of the 6 cases of portal cirrhosis, 5 were positive and one was doubtful. Of 10 cases of longstanding chronic passive congestion due to cardiac failure, 4 were positive and 2 doubtful.

In Group III there were 9 cases of obstructive jaundice. Two were strongly positive. In one of these the test was taken just before exitus, after many months of increasing jaundice. The other was in a case where the jaundice was of only three weeks' duration; at operation a carcinoma of the pancreatic head was found. These results are compatible with those of Pohle and Stewart,<sup>3</sup> who found that the flocculation test was positive in 18 of 23 cases of obstructive jaundice. This may be explained on the basis of damage to the liver, which occurs with long-standing biliary obstruction or with ascending infection and hepatic damage accompanying obstruction.

TABLE I

|  | 0<br>NEGATIVE | 1-2 PLUS<br>DOUBTFUL | 3-4 PLUS<br>POSITIVE |
|--|---------------|----------------------|----------------------|
| I. No liver disease or biliary obstruction | 67            | 12                   | 4                    |
| Cholecystitis                              | 10            | 2                    | 0                    |
| Hyperthyroidism                            | 3             | 3                    | 2                    |
| Others                                     | 54            | 7                    | 2                    |
| II. Intrinsic liver disease                | 4             | 4                    | 14                   |
| Catarrhal jaundice                         | 0             | 0                    | 4                    |
| C.P.C. of liver                            | 1             | 2                    | 4                    |
| Portal cirrhosis                           | 0             | 1                    | 5                    |
| Others                                     | 0             | 1                    | 1                    |
| III. Obstructive jaundice                  | 7             | 0                    | 2                    |
| Carcinoma of head of pancreas              | 5             | 0                    | 2                    |
| Common duct stone                          | 2             | 0                    | 0                    |

## CONCLUSIONS

We feel that the cephalin-cholesterol flocculation test is of definite use in the evaluation of clinical jaundice and hepatic disease. It is no more infallible than any other test of liver status. However, in doubtful cases and in "false" positives, there is usually a factor which makes the existence of subclinical hepatic damage or irritation (by other testing methods) a possibility.

That the test is exceedingly inexpensive and simple to perform makes its value greater, and we suggest that this test be used routinely in the study of cases with jaundice or with suspected hepatic disease.

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# RETENTION OF NONPROTEIN NITROGEN IN THE BLOOD OF THE RABBIT FOLLOWING INTRAVENOUS INJECTIONS OF A HYPERTONIC SOLUTION OF DEXTROSE\*

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RENAL lesions have been observed in both man and experimental animals following the intravenous injection of hypertonic solutions of suerose.<sup>1-3</sup> Recently Rigdon and Cardwell<sup>4</sup> studied these lesions and found that the primary changes in the kidney occurred in the epithelial cells in the convoluted portion of the tubules. These cells were swollen, and their cytoplasm stained very lightly with eosin. The nucleus was shrunken, and the periphery was serrated. There was a retention of nonprotein nitrogen in the blood of the rabbits showing these histologic changes.

Investigators have usually found it difficult to produce renal lesions by the intravenous injection of hypertonic sucrose with any degree of regularity.<sup>1, 2</sup> Rigdon and Cardwell<sup>4</sup> observed that many of their clinical cases were dehydrated when they were first given the hypertonic solution of suerose. In view of this clinical observation, these investigators dehydrated rabbits before and during the period in which the suerose was given. With this technique they were able to produce renal lesions routinely. Accompanying this lesion there was a retention of nonprotein nitrogen in the blood.

It was suggested by Rigdon and Cardwell<sup>4</sup> that this renal lesion resulted from a retention of suerose and water in the cytoplasm of the epithelial cells in the convoluted portion of the renal tubules. The nuclei of these cells were shrunken as the result of dehydration. The swollen epithelial cells sometimes completely blocked the lumina of the tubules. This process by which retention of nonprotein nitrogen occurred following the intravenous injection of a hypertonic solution of sucrose was reversible as shown by a diminution in the quantity of nonprotein nitrogen that was present in the blood of these rabbits following the addition of water and cabbage to their diet.

In the present experiments a hypertonic solution of dextrose was given intravenously to rabbits to study its effect on the kidney and on the retention of nonprotein nitrogen in the blood. The technique was the same as that used in previous studies when a hypertonic solution of sucrose was given to rabbits.<sup>4</sup>

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The nonprotein nitrogen determination was made in the chemistry laboratory of the department of medicine. I wish to thank Dr. Diggs for the use of his laboratory facilities.

TABLE I  
RETENTION OF NONPROTEIN NITROGEN IN THE BLOOD OF RABBITS GIVEN A 50.0 PER CENT SOLUTION OF DEXTROSE INTRAVENOUSLY

| QUANTITY OF NONPROTEIN NITROGEN IN THE BLOOD (MG. PER 100 C.C.) |      |                  |     |     |     |      |      |      |      |      |       |                      |     |      |     |                 |      |      |     |     |     |     |     |
|---|------|------------------|-----|-----|-----|------|------|------|------|------|-------|----------------------|-----|------|-----|-----------------|------|------|-----|-----|-----|-----|-----|
| EXP.<br>DAY   |      | OATS             |     |     |     |      |      |      |      |      |       | OATS, CABBAGE, WATER |     |      |     |                 |      | OATS |     |     |     |     |     |
|   |      | 800              | 801 | 802 | 803 | 808  | 809  | 900  | 901  | 928  | 929   | 930                  | 931 | 978  | 979 | 902             | 903  | 933  | 934 | 935 | 806 | 807 | 980 |
| 1   |      |                  |     |     |     |      |      |      | 33   | 37   |       |                      |     |      |     |                 | 35   | 33   |     |     |     |     |     |
| 3   | 121* | 53               | 44* | 67  | 86  | 75   | 60   | 67   | 121  | 69.5 | 80.5* | 69.5                 | 55  | 60   | 50  | 67              | 52   | 54   | 42  | 50  | 67  | 50  |     |
| 4   |      | 88               |     |     | 78  | 78   | 92   | 75   | 148† | 107  |       | 87.5†                |     | 113* | 60  | 67              | 67.5 | 58.5 | 52  | 60  | 57  |     |     |
| 4   |      | 120 <sup>b</sup> |     |     |     |      |      |      |      |      |       |                      |     |      |     |                 |      |      |     |     |     |     |     |
| 5   |      |                  |     |     |     |      |      |      |      | 229* |       |                      | *   |      |     |                 | 47*  |      |     |     |     |     |     |
| 6   |      |                  |     |     | 78* | 120  | 115  | 150  | 92   |      |       | 72                   |     |      | 75  | 67              | 58   | 58.5 |     | 60  | 60  |     |     |
| 7   |      |                  |     |     |     | 171* | 120† | 300* | 71.5 |      |       | 67.5                 |     |      | 63  | 67              | 48   | 44   |     | 55† | 60  |     |     |
| 7   |      |                  |     |     |     |      |      |      |      |      |       |                      |     |      |     | 60 <sup>c</sup> |      |      |     |     | 63* |     |     |
| 10  |      |                  |     |     |     |      |      |      | 42   |      |       | 37                   |     |      | 40  |                 |      | 34   | 38  |     | 43  |     |     |
| 11  |      |                  |     |     |     |      |      |      | 34   |      |       | 33                   |     |      |     |                 |      | 27   | 32* |     |     |     |     |
| 17  |      |                  |     |     |     |      | 40   |      |      |      |       |                      |     |      | 38  |                 |      |      |     | 33  |     |     |     |

All rabbits given only oats for three days before experiment begun.

\*Rabbits either died or were killed at this time.

†Rabbits given cabbage, water, and oats at this time to see if the N.P.N. would return to normal.

‡Rabbits 800, 801, 802, 803, 928, 929, 930, 931, 933, 934, 935, and 979 received 25 c.c. of dextrose each day for three successive days.

Rabbits 808, 809, 900, 901, and 978 received 25 c.c. of dextrose each day for five successive days.

Rabbits 806, 807, and 980 were controls; they received no dextrose.

## METHODS AND MATERIALS

Normal adult rabbits were used. They were given only oats for three days. At this time one group was given water, cabbage, and oats. A second group was kept on a diet of only oats. All of the rabbits were given 25.0 c.c. of a 50.0 per cent buffered solution of dextrose\* (*d*-glucose) intravenously each day for three to five days. The controls were given only oats and no dextrose during the time of the experiment.

The nonprotein nitrogen and urea nitrogen content of the blood were determined by standard methods at intervals during the experiment. The rabbits were killed at varying times, and the kidneys were placed immediately into a solution of 10.0 per cent formalin. Paraffin sections were made and were stained with hematoxylin and eosin.

The rabbits were weighed at frequent intervals. When some of the rabbits, given only oats and dextrose, showed a retention of nonprotein nitrogen, they were given cabbage and water. This procedure was used for those rabbits in which the function of the kidney was permitted to return to normal as indicated by the level of nonprotein nitrogen in the blood.

## EXPERIMENTAL DATA

Fourteen rabbits were fed only oats and were given dextrose intravenously. Each of the animals that lived for either four days or longer showed a retention of nonprotein nitrogen in the blood. Rabbits 900, 928 and 931, as shown in Table I, were given cabbage and water after they developed a retention of nonprotein nitrogen. The nonprotein nitrogen in Rabbit 900 was 120 mg. per 100 c.c. on the seventh day and on the seventeenth day of the experiment it had returned to within the range of normal. The nonprotein nitrogen in Rabbit 928 was 148 mg. per 100 c.c. on the fourth day of the experiment, and the nonprotein nitrogen in Rabbit 931 was 87.5 mg. per 100 c.c. On the eleventh day of the experiment the former rabbit's nonprotein nitrogen was 34 mg. per 100 c.c., and the latter's was 33 mg. per 100 c.c.

Five rabbits as shown in Table I were given cabbage, oats and water during the period in which the hypertonic solution of dextrose was injected. There was only a slight rise in the level of the nonprotein nitrogen in any of these rabbits. Rabbits 902 and 903 showed the greater degree of retention. They did not receive adequate amounts of cabbage during the time of the experiment.

Three rabbits (Table I) were given only oats, and the nonprotein nitrogen was determined at frequent intervals. The highest level of the nonprotein nitrogen was 67 mg. per 100 c.c. on the third day in Rabbit 807 and 60 mg. per 100 c.c. in Rabbit 806 on the sixth day. The results of this experiment illustrate the effect of dehydration on the retention of nonprotein nitrogen in the blood of the rabbit.

Urea nitrogen determinations were made in Rabbits 978, 979, and 980. They were 25.0 mg. per 100 c.c. in Rabbit 978 on the second day of the experiment, 28.0 mg. per 100 c.c. in Rabbit 979, and 22.0 mg. per 100 c.c. in Rabbit

\*The dextrose was supplied by Eli Lilly and Company.

980. The urea nitrogen was 50.0 mg. per 100 c.c. in Rabbit 978 on the third experimental day. It was 60.0 mg. per 100 c.c. in Rabbit 978 on the fourth and only 30.0 mg. per 100 c.c. in the control, Rabbit 980. Rabbit 980 had only 25.0 mg. per 100 c.c. urea nitrogen on the fiftieth day. These observations show that the urea nitrogen of the blood increases in the rabbit following the intravenous injection of a 50.0 per cent solution of dextrose when the animals are dehydrated.

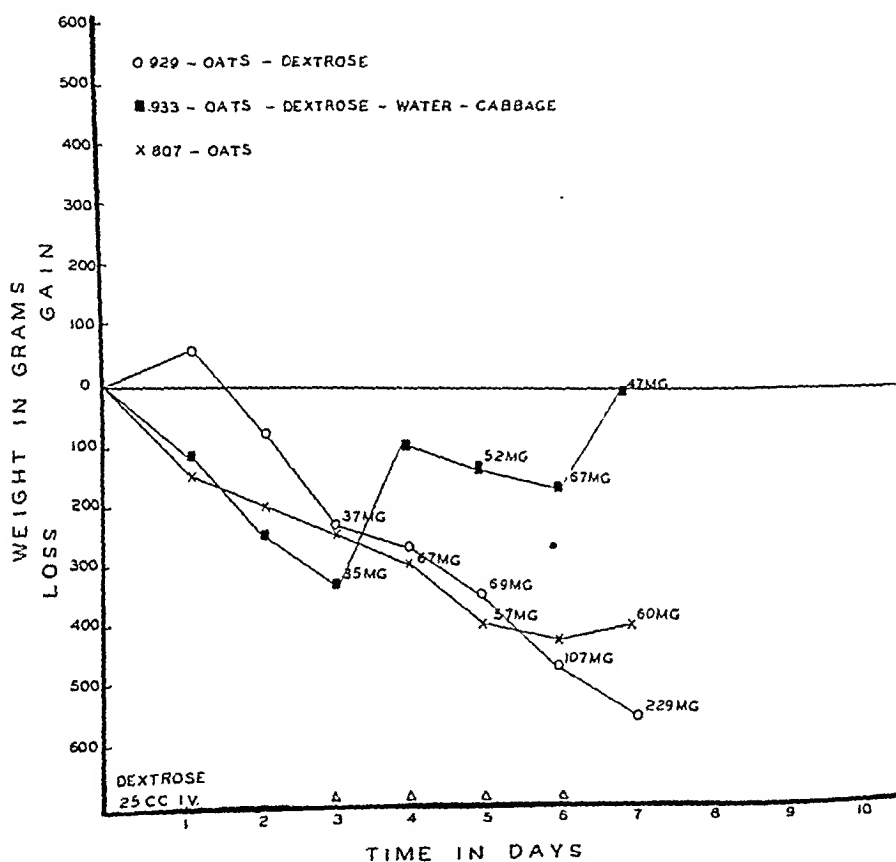


Fig. 1.—The graph illustrates the loss in weight and the amount of nonprotein nitrogen in the blood of the rabbits at different intervals during the experiment.

All of the rabbits lost weight during the time of the experiment. The average decrease in weight of the group given cabbage, water, oats, and the hypertonic solution of dextrose was 60 Gm. Those given only oats lost an average of 389 Gm. Fig. 1 illustrates the loss in weight of the rabbits and the amount of nonprotein nitrogen in the blood at varying intervals during the experiment.

Pathologic studies were made on the kidneys from Rabbits 801, 807, 903, 929, 933, 935, 978, and 979. Each of these animals was killed. The kidneys from Rabbits 809 and 901 were also studied histologically. These rabbits were dead for two hours or less before the kidneys were removed. There were no macroscopic lesions observed in the kidneys. The epithelial cells in the con-

voluted portion of the tubules in Rabbit 929 were slightly swollen and the cytoplasm was filled with small pink granules. A small amount of albumin was present in the lumina of the tubules. The kidney from Rabbit 807 showed essentially the same characteristics as the kidney from Rabbit 929. The epithelial cells in the kidney of Rabbit 979 were swollen, and the cytoplasm stained lightly with eosin. The kidneys from the other rabbits showed only some swelling of the epithelial cells in the convoluted portions of the tubule. In fact, there was very little histologic difference in any of these sections. The kidneys from the rabbits showing a retention of nonprotein nitrogen were very similar to the controls and also to those in which the level of nonprotein nitrogen was elevated and then returned to normal.

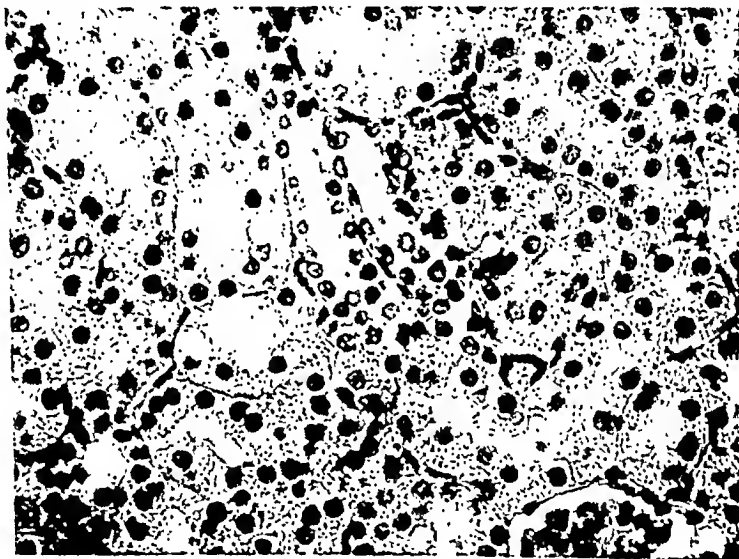


Fig. 2.—Rabbit 979. The epithelial cells in the convoluted portions of the tubules are swollen. Usually the cytoplasm is finely granular and stains deeply with eosin. This rabbit was given 25.0 c.c. of a 50.0 per cent solution of dextrose daily for three days. The nonprotein nitrogen on the fourth day at the time of death was 113 mg. per 100 c.c.

#### DISCUSSION

The data obtained from this study show that rabbits given 3 to 5 intravenous injections of a hypertonic solution of dextrose (25.0 c.c. of a 50.0 per cent solution) develop a retention of nonprotein nitrogen when the intake of fluid is restricted. Following the retention of the nonprotein nitrogen, if the animals are given fluids in the form of water and cabbage, the level of the nonprotein nitrogen in the blood rapidly returns to normal. This observation indicates that the mechanism by which the nonprotein nitrogen is retained in the blood is a reversible process. We are unable to give the maximum level which nonprotein nitrogen may reach and still return to normal.

No characteristic pathologic changes were observed in the kidneys of those rabbits that developed a retention of nonprotein nitrogen following the intravenous injection of a hypertonic solution of dextrose. The epithelial cells of the convoluted portion of the tubules may become slightly swollen and the cytoplasm may become granular. Small amounts of albumin were present in

a few of the tubules. The kidneys from the rabbits given oats and those from the rabbits given dextrose, oats, water, and cabbage showed essentially the same histologic changes.

The rabbits given hypertonic solutions of sucrose had swollen and vacuolated epithelial cells in the convoluted portion of the renal tubules. The nuclei of the epithelial cells were shrunken.<sup>4</sup> It was interesting to observe the difference in the histologic changes in the kidney in the rabbits given the hypertonic solution of sucrose and in those given the hypertonic solutions of dextrose. There was, however, a retention of the nonprotein nitrogen in the blood of the rabbit following the intravenous injection of both of these sugars.

Oats were selected for the basic diet of these rabbits since the amount of fluid obtained from them is only negligible. Cabbage was used primarily for its high-water content.

It would be interesting to know if there are any clinical complications following the use of hypertonic solutions of dextrose similar to those observed following the intravenous injection of hypertonic solutions of sucrose. Hypertonic solutions of dextrose are frequently used clinically for the reduction of intracranial pressure. Masserman<sup>5</sup> observed the effect of various amounts and different concentrations of dextrose on 85 normal patients. He found that "the administration of 50 Gm. or less in 20 per cent solution produced no untoward clinical sequelae other than diuresis; however, the intravenous injection of 100 Gm. or more in 30 to 50 per cent solution caused headaches and other adverse symptoms in 72 per cent of cases, whereas 58 per cent of the patients receiving 185 Gm. or more suffered transient pyrexia." Blau<sup>6</sup> has suggested that a hypertonic saline solution is preferable to hypertonic dextrose to reduce cerebrospinal pressure.

#### SUMMARY

Rabbits when dehydrated and given a daily injection of 25.0 c.c. of a hypertonic solution of dextrose for three to five days developed a retention of nonprotein nitrogen and urea nitrogen in the blood.

Following the retention of nonprotein nitrogen, when fluids were given, the nonprotein nitrogen content of the blood returned to normal.

There were no characteristic pathologic lesions observed in the kidney of the rabbits given solutions of hypertonic dextrose and developing a retention of nonprotein nitrogen.

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# CONCENTRATION OF SULFAGUANIDINE (SULFANILYLGUANIDINE) IN THE PERIPHERAL BLOOD OF HUMAN BEINGS AFTER ITS INTRODUCTION INTO THE LARGE BOWEL AND AFTER ORAL ADMINISTRATION\*

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SULFAGUANIDINE (sulfanilylguanidine), a sulfonamide derivative, has been described by Marshall and his associates<sup>1</sup> as a chemotherapeutic agent for intestinal infections. Relatively small amounts of the drug are absorbed from the gastrointestinal tract, thus providing a rather high concentration in the intestine where it may act on the coliform bacilli of the intestinal flora. It has been shown<sup>1</sup> that in dogs sulfaguanidine is absorbed to a much greater degree by the small bowel than by the large bowel and that the levels of the sulfaguanidine in the blood after absorption from the large bowel are much lower than the levels of sulfanilamide when the latter drug is given by the same route.

Five patients with well-functioning colostomies and with clinically and grossly normal gastrointestinal tracts above the site of obstruction afforded the opportunity of comparing the blood levels of sulfaguanidine which might result from its oral administration with those found after the drug was instilled into the large bowel in human beings. The procedure to be described was carried out after the colon had emptied spontaneously in the usual manner and at a time when no further activity of the large bowel was expected for at least six to eight hours. Doses of 3 to 5 Gm. (60 or 100 mg./kg. of body weight) of sulfaguanidine† were used in the form of suspensions and were introduced into the large bowel in a manner not liable to initiate peristalsis. The suspension was introduced into the distal loop of a transverse colostomy in 2 patients, into the proximal loop of a sigmoid colostomy in 2 patients, and into the distal loop of a sigmoid colostomy in one patient. Blood samples were collected at intervals during the following four to six hours. At the end of these periods of sampling, the dressing covering the colostomy was inspected to detect evidence that the suspension had been expelled. The loop of large bowel was then flushed with warm water to remove as much of the drug as possible. A dose of the drug similar to that placed in the large bowel was then given by mouth, and blood samples were collected at intervals during the subsequent seventeen hours. The levels of free sulfaguanidine in the blood samples were determined according to the method described by Marshall and his co-workers.<sup>2</sup>

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†Sulfaguanidine through the courtesy of Lederle Laboratories, New York.

The results are shown in Table I, and the trends of the concentration of the drug in the blood are represented in Fig. 1. There was no evidence that the drug had been expelled from the colon, yet a measurable concentration of sulfaguanidine was not found in the peripheral blood of any of the 5 patients during the four to six hours following its introduction into the large bowel. Concentrations

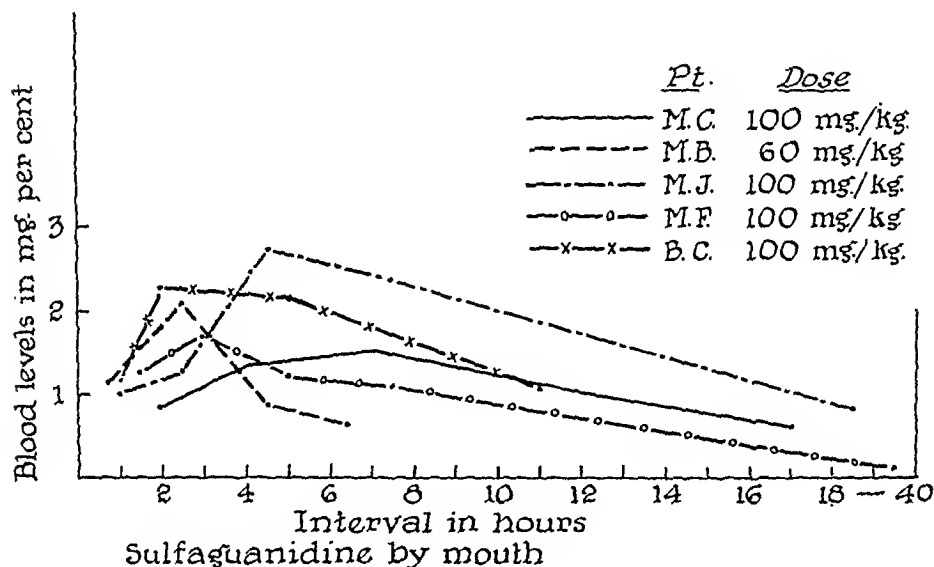


Fig. 1.—Sulfaguanidine levels in the peripheral blood after oral administration.

TABLE I

BLOOD LEVELS OF SULFAGUANIDINE AFTER ITS INTRODUCTION INTO THE LARGE BOWEL AND AFTER ORAL ADMINISTRATION

| PATIENT                    | M. B.      |       | M. C.       |       | M. F.       |       | M. J.       |       | B. C.       |       |
|----------------------------|------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|
| Large Bowel                |            |       |             |       |             |       |             |       |             |       |
| DOSE                       | 60 MG./KG. |       | 100 MG./KG. |       | 100 MG./KG. |       | 100 MG./KG. |       | 100 MG./KG. |       |
|                            | HR.        | MG. % | HR.         | MG. % | HR.         | MG. % | HR.         | MG. % | HR.         | MG. % |
|                            | 1          | 0.0   | 1½          | 0.0   | 1½          | 0.0   | 2           | 0.0   | 1           | 0.0   |
|                            | 3          | 0.0   | 3½          | 0.0   | 4½          | 0.0   | 5           | 0.0   | 3           | 0.0   |
|                            | 5          | 0.0   | 5½          | 0.0   |             |       |             |       | 4           | 0.0   |
| After Oral Administration* |            |       |             |       |             |       |             |       |             |       |
| DOSE                       | 60 MG./KG. |       | 100 MG./KG. |       | 100 MG./KG. |       | 100 MG./KG. |       | 100 MG./KG. |       |
|                            | HR.        | MG. % | HR.         | MG. % | HR.         | MG. % | HR.         | MG. % | HR.         | MG. % |
|                            | 2          | 0.89  | 1           | 1.18  | 2           | 1.19  | 2           | 0.90  | 1           | 1.14  |
|                            | 4          | 1.42  | 2           | 2.13  | 4           | 1.60  | 4           | 1.37  | 3           | 2.28  |
|                            | 7          | 1.60  | 5           | 0.94  | 6           | 1.20  | 6           | 2.62  | 5           | 2.05  |
|                            | 12         | 1.06  | 7           | 0.70  | 8           | 1.02  | 9           | 2.40  | 11          | 1.14  |
|                            | 17         | 0.71  | 10          | 0.00  | 18          | 0.23  | 18          | 0.91  |             |       |
|                            |            |       | 17          | 0.00  |             |       |             |       |             |       |

\*Sulfaguanidine removed from colon by lavage.

of the free form of the drug, varying from 0.89 to 2.13 mg. per cent, were found in the peripheral blood in all 5 of the samples withdrawn two hours after oral administration. The peaks of concentration occurred within two to seven hours, and the maximal blood level was 2.62 mg. per cent. In general, the blood levels corresponded to the dosage, being higher when larger amounts of the drug were given.



Following the oral administration of the drug, one patient complained of vertigo which lasted for twenty-four hours and another was nauseated for six hours. In 3 of the patients erythrocyte and leucocyte counts and hemoglobin determinations were made at the beginning and at the end of the experimental period. No significant differences were found.

#### CONCLUSION

Sulfaguanidine, in the form of an aqueous suspension, when introduced directly into the large bowel through a colostomy stoma, is not absorbed from the left half of the colon in human beings.

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### THE IMMEDIATE EFFECT OF CIGARETTE SMOKING UPON BASAL METABOLIC RATES OF UNIVERSITY MEN AND WOMEN\*

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THE object of this investigation was to determine the immediate effect of cigarette smoking upon the basal metabolic rate of the smoker, since a question as to the existence of such an effect had arisen. In spite of a careful search of the literature, little definite evidence was found. There seems to be, however, a general impression among clinical workers that smoking is followed by a rise in basal metabolism. We resolved to test this assumption under conditions approaching those under which clinical determinations are made. It was hoped that a definite answer to the question of the immediate effect of smoking could be obtained from the results.

Males<sup>1</sup> noted that the injection of rats with minimal doses of nicotine, the active principle of cigarette smoke, produced a transient rise in metabolic rate, followed by a more prolonged period of subnormal metabolic activity. This may be taken as an indication that smoking is capable of affecting the metabolism in some way. Furthermore, Sehlumm<sup>2</sup> found a high basal metabolic rate (average +19.9 per cent by Harris-Benedict standards) in heavy smokers in Germany; the basal metabolic rates fell to a normal level soon after smoking was discontinued. More recently, Hiestand, Ramsey, and Hale<sup>3</sup> reported further evidence for an increase in basal metabolism as an immediate result of cigarette smoking. There is, therefore, some indication in the literature that smoking may affect the metabolic rate.

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## EXPERIMENTAL

The subjects taken for these tests were men and women of college age, selected at random from students apparently in good physical condition at the time of the experiment. All were accustomed to smoking either habitually or occasionally. The subject relaxed for one-half hour before the first test; the control basal rate was then determined, using the Benedict-Roth recording metabolism apparatus. Two six-minute tests were averaged for the control run. Following this, the subject was allowed to sit up and smoke approximately three-fourths of a cigarette. Physical exertion was kept at a minimum. The subject then lay down again for another rest period of twenty-five to thirty minutes, at the end of which time two more six-minute runs were taken. From the average of these, the apparent basal metabolic rate after smoking was determined. The deviation from the control test, previously obtained, was expressed as percentage difference. The calculations of the normal calories per hour were made by use of the standards of Dubois, as modified by Sandiford and Boothby.

TABLE I

| SUBJECT    | SEX* | SMOKING HABITS† | BASAL RATE PER CENT | NORMAL CAL./HR. | ACTUAL CAL./HR. (CONTROL) | AFTER SMOKING CAL./HR. | PER CENT DEVIATION |
|------------|------|-----------------|---------------------|-----------------|---------------------------|------------------------|--------------------|
| V. Bu.     | F    | H               | - 2.5               | 56.8            | 55.4                      | 64.1                   | +15.7              |
| M. C.      | F    | O               | -10.0               | 54.2            | 48.8                      | 55.9                   | +14.6              |
| A. T.      | F    | O               | - 9.8               | 59.0            | 53.2                      | 59.4                   | +11.7              |
| S. C.      | F    | O               | -10.6               | 56.5            | 50.5                      | 55.5                   | + 9.9              |
| F. J.      | F    | O               | -14.1               | 59.0            | 50.7                      | 53.4                   | + 5.3              |
| J. W.      | M    | O               | -15.6               | 74.6            | 63.0                      | 66.2                   | + 5.1              |
| J. V.      | M    | H               | -13.5               | 70.9            | 61.3                      | 64.0                   | + 4.4              |
| D. F.      | F    | O               | - 8.9               | 60.9            | 55.5                      | 57.3                   | + 3.2              |
| J. O.      | M    | H               | -15.9               | 66.8            | 56.2                      | 57.9                   | + 3.0              |
| S. L.      | M    | H               | -14.6               | 78.1            | 66.7                      | 67.9                   | + 1.8              |
| E. B.      | F    | O               | - 8.8               | 53.5            | 48.8                      | 49.7                   | + 1.8              |
| D. C.      | F    | H               | -17.5               | 59.0            | 48.7                      | 49.3                   | + 1.2              |
| J. C.      | F    | H               | - 3.4               | 58.3            | 56.3                      | 56.3                   | 0.0                |
| E. S.      | F    | H               | - 6.6               | 58.8            | 54.9                      | 54.0                   | - 1.6              |
| J. F.      | M    | H               | - 7.9               | 78.7            | 72.5                      | 70.6                   | - 2.6              |
| M. R.      | F    | O               | - 4.7               | 55.0            | 52.4                      | 50.7                   | - 3.2              |
| M. S.      | F    | H               | - 5.0               | 58.3            | 55.1                      | 53.5                   | - 3.4              |
| D. U.      | M    | H               | - 3.6               | 75.4            | 72.7                      | 69.4                   | - 4.5              |
| V. Be.     | F    | H               | - 0.8               | 59.0            | 58.5                      | 54.1                   | - 7.5              |
| J. G.      | M    | H               | -14.3               | 72.9            | 62.5                      | 57.3                   | - 8.7              |
| Mean value |      |                 |                     |                 |                           |                        | + 2.3              |

\*M = Male.

F = Female.

†O = Occasional smoker.

H = Habitual smoker.

Standard precautions and procedures (i.e., testing for leaks, determining absorption of carbon dioxide, etc.) were used throughout the experiment. The subject was, of course, in basal condition and had not smoked on the morning of the tests. In order to control a factor which might possibly vary, the same brand of cigarettes was used in all cases. It was shown at one time by the Bureau of Investigation of the American Medical Association<sup>4</sup> that this brand contained 1.44 per cent nicotine, which was slightly below the average for standard brands. The necessity for this precaution is indicated by the work of

Haag,<sup>5</sup> who found that the blood pressure of anesthetized dogs, given intravenous injections of smoke solutions prepared from several brands of cigarettes, could be correlated with the nicotine content of the cigarettes.

The 20 subjects were selected from more than 40 upon whom control tests were run. Only those tests in which, by all criteria, the subjects appeared relaxed, and in which the oxygen consumption in the two six-minute periods agreed within 3 mm. on the chart were considered. Results of doubtful validity were not used.

The results of the tests are shown in Table I. The last column was obtained by dividing the difference between the two previous columns by the control (actual calories per hour) and multiplying by 100.

#### DISCUSSION

The results given here shown that the apparent basal metabolic rate determined approximately one-half hour after smoking may be either above or below the control test taken prior to smoking, but that the deviation is by no means constant. In many cases the difference obtained was certainly great enough to be considered significant.

The work of many investigators has indicated that smoking, or nicotine, stimulates the adrenal glands to an increased secretion of adrenaline. Houssay and Molinelli<sup>6,7</sup> showed that nicotine acts directly upon the adrenal medulla of dogs, causing release of the hormone by a specific reaction with that tissue. Haggard and Greenberg<sup>8</sup> found a rise in blood sugar after smoking, which they attributed to the action of adrenaline. Johnson and Short<sup>9</sup> observed a rise in the peripheral skin temperature after smoking, while Short and Johnson<sup>10</sup> noted that pulse rate, blood sugar, peripheral skin temperature, and blood pressure were similarly influenced by smoking and by small amounts of adrenaline.

In the recent report of Hiestand, Ramsey, and Hale<sup>3</sup> an adrenaline effect was pointed out as the probable cause of the physiologic responses to smoking. In 82 per cent of their subjects (not in basal condition) the metabolic rate was increased following smoking. Sixty per cent of our subjects showed an increased basal metabolic rate, whereas 35 per cent showed a lowering in the rate. The mean value of the percentage deviation was  $\pm 2.3$  in our study, while the above authors reported an average of  $+8.9$ . They did not attempt to explain the lowering of the metabolic rate, immediately following smoking, in 13 per cent of their subjects.

As Haggard and Greenberg<sup>8</sup> showed, when the blood sugar is raised, an increment in carbohydrate oxidation, and, therefore, a higher metabolic rate was to be expected. For this reason, a rise in basal metabolism following smoking was anticipated. However, a possible explanation for the unexpected variation in results has been tentatively advanced. It is well known that the sympathetic nervous system varies in responsiveness in different individuals, and that the adrenals are under its control. It is suggested that the response of these glands to the stimulation due to smoking occurs after different time intervals in different subjects, causing a preliminary rise in basal metabolic rate, followed by a drop

to subnormal values before the return to normal. This supposition is borne out by the work of Males<sup>1</sup> on rats. Johnson and Short<sup>9</sup> noted a time difference in their subjects in changes of the peripheral skin temperature after smoking, and Hiestand, Ramsey, and Hale<sup>3</sup> noted changes in responses of their subjects over a period of forty-five minutes following smoking, with regard to heart rate, blood pressure, oxygen pulse, and metabolic rate.

Haggard and Greenberg<sup>8</sup> observed that the respiratory quotient reached its peak in about fifteen minutes after smoking, and during the next thirty minutes returned to the normal level or slightly below. As the method of indirect calorimetry was used in our determinations, it is evident that in cases of apparent increase in the basal metabolic rate, the actual increases may have been even greater than those calculated, since the method of calculation assumes a respiratory quotient of exactly 0.82. The work of Dill, Edwards, and Forbes<sup>11</sup> on ten subjects, however, indicated that the smoking of one cigarette resulted in no appreciable change in respiratory quotient.

With regard to interpreting our own results, therefore, it is possible that (1) the "metabolic rate-time curve" may have been distinct and characteristic for each person; and (2) the basal metabolic rates of our subjects were determined at different points on their individual curves.

With regard to other phenomena observed, it was found that the pulse rate was generally higher, the body temperature usually higher or constant, and the rate of respiration often higher after smoking. This is in general agreement with the work of Short and Johnson and others, although Hiestand, Ramsey, and Hale found a decrease in the rate of respiration. It was further noted that sex seemed to have no influence upon the immediate reaction to smoking.

It was significant that all the control basal metabolic rates of our subjects showed a negative deviation from the predicted standards. This is in general accord with the results of Martin,<sup>12</sup> Mason,<sup>13</sup> and Tilt and Walters,<sup>14</sup> obtained upon subjects in tropical or subtropical climates.

#### SUMMARY

1. The apparent basal metabolic rate after smoking generally shows an appreciable deviation from a control rate determined prior to smoking.
2. The generally accepted rise in basal metabolism some time after smoking is by no means a universal phenomenon.
3. A rise in pulse rate, respiration rate, and body temperature following smoking is generally observed.
4. The sex of the subject apparently does not influence his energy output following smoking.
5. A possible explanation of the individual response to smoking is offered.

Grateful acknowledgment is made to our voluntary subjects for their faithful cooperation, and to Dr. William C. Boeck, of Los Angeles, for his helpful suggestion with regard to interpretation of our data.

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# LABORATORY METHODS

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## GENERAL

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### SLIDE AGGLUTINATION IN DETECTING PERTUSSIS ANTIBODIES\*

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#### COMPARISON OF SLIDE WITH TUBE AGGLUTINATION

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DIAGNOSIS by means of slide agglutination has been used successfully for some time in Brucellosis,<sup>1-6</sup> and in typhoid-paratyphoid infections.<sup>7, 8</sup> More recently Evans and Maitland<sup>9</sup> recommended slide agglutination for the diagnosis of pertussis. Using a concentrated antigen of live *H. pertussis* and examining the slides microscopically, they obtained more positive results on the slide than on macroscopic tube agglutination.

The main advantages of slide over tube agglutination are (1) the small amount of serum required for the test, (2) the rapidity of the reaction (agglutination usually occurs within a few minutes) and (3) the simple apparatus necessary to carry out the test. Therefore, if on further tests slide agglutination should prove sufficiently sensitive to detect weakly positive sera, it would be an improvement on the more cumbersome and time-consuming method of tube agglutination. With this object in view we have tested a fairly large number of sera by slide and tube agglutination.

The use of a live antigen in slide agglutination as recommended by Evans and Maitland<sup>9</sup> seemed undesirable, since it would be necessary to prepare a suspension of the bacilli and standardize it for each day's tests. We found, after several trials, that we could use with equal success a suspension that was killed by means of 0.5 per cent formalin, or 1:50,000 dilution of merthiolate. This antigen did not lose its agglutinability after seven months' storage, which was the period of this investigation. Antigens that were killed by means of gentian violet in a 1:20,000 dilution or brilliant green in a 1:40,000 dilution became less agglutinable upon storage and were therefore not used. The antigen that we employed in our tests was prepared as follows:

A forty-eight-hour growth of *H. pertussis* on freshly prepared Bordet-Gengou medium was emulsified in sterile physiologic saline. The suspension was filtered through sterile cotton to remove particles of agar, and the bacteria were precipitated by centrifugation. They were washed twice in saline, and after the second washing they were suspended in physiologic saline which contained 1:50,000 merthiolate. The antigen was stored at 8 to 10° C.

The optimum concentration of the antigen for rapid slide agglutination was determined by titration with an antipertussis rabbit serum and was later

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checked with known positive human sera. Serial dilutions were made of the positive serum, and each dilution was tested with various concentrations of the antigen. As a control a live suspension of the bacilli was used in a concentration of 100 billion as recommended by Evans and Maitland. We found that a concentration of 25 billion bacilli per milliliter of the killed antigen gave more clear-cut agglutination than higher concentrations, and agglutination took place in the same dilutions as with the live antigen. We therefore used our antigen in a 25 billion concentration.

Before each day's test, the antigen was diluted with sterile physiologic saline to 25 billion bacilli per milliliter and was filtered through a 60-mesh monel metal wire gauze to remove clumps. It was then titrated with the positive serum to determine its sensitivity on the day of the test. The same positive serum was used throughout this study, and its titer remained remarkably constant.

*The Test.*—The sera were tested undiluted; 0.01, 0.02, 0.04, and 0.08 ml. amounts were used according to the method of Welch-Stuart<sup>7</sup> for typhoid-paratyphoid diagnosis. The stated amounts of serum were measured into the center of 4 paraffin rings on a glass slide by means of a pipette that was graduated into 0.01 ml. A drop of the antigen was added to the serum from a standardized capillary pipette that delivered approximately 0.015 ml. in each drop. The serum and antigen were thoroughly mixed by means of a wooden applicator, beginning with the smallest amount of serum. The slide was tilted back and forth for one minute, and a preliminary reading was made. The final reading was made after an additional three minutes' incubation at room temperature. All readings were made macroscopically, using a constant light. We found it unnecessary to read the agglutination on the slide microscopically since the clumps were distinctly visible to the naked eye.

Strongly positive sera agglutinated usually within one minute; weakly positive sera required two to three minutes. Where agglutination was complete, the clumps were large and the fluid clear. A serum was considered negative when it showed incomplete or no agglutination after four minutes' incubation at room temperature; a longer period of incubation could not be used, as the mixtures began to dry after four minutes.

Agglutination usually took place first in the 0.01 and 0.02 ml. then in the 0.04 and 0.08 ml. amounts of the serum, thus showing that the optimum proportion of serum to antigen was in the 0.01 or 0.02 ml. amounts of the serum. The final results were recorded on the basis of agglutination in 0.01 ml. of the serum. When agglutination was complete in the undiluted serum, serial dilutions were made, and 0.01 ml. of each dilution was tested for agglutination. The result of the titration was recorded in terms of the highest dilution of the serum that showed distinctly visible clumps.

#### TUBE AGGLUTINATION

*Antigen.*—The preparation of the antigen was described in detail in a previous publication.<sup>10</sup> A formalinized saline suspension of a forty-eight-hour growth of *H. pertussis* on Bordet-Gengou medium was standardized and was tested for agglutination with a known positive serum. It was stored in con-

TABLE I  
SLIDE VERSUS TUBE AGGLUTINATION IN DETECTING PERTUSSIS ANTIBODIES

| HISTORY  | TOTAL SERA | AGGLUTINATION |       |      | TOTAL POSITIVE |      |
|--|------------|---------------|-------|------|----------------|------|
|  |            | NO.           | SLIDE | TUBE | SLIDE          | TUBE |
| Persons vaccinated against pertussis                                   | 19         | 19            | +     | +    | 19             | 19   |
| Mice vaccinated with various pertussis antigens                        | 23*        | 16            | +     | +    | 19             | 16   |
|  |            | 3             | +     | 0    |                |      |
|  |            | 4             | 0     | 0    |                |      |
| Normal mice  | 3†         | 3             | 0     | 0    | 0              | 0    |
| Persons with history of whooping cough                                 | 30         | 2             | +     | +    | 8              | 2    |
|  |            | 6             | +     | 0    |                |      |
|  |            | 22            | 0     | 0    |                |      |
| Persons with colds or coughs definitely exposed to whooping cough      | 50         | 6             | +     | +    | 14             | 7    |
|  |            | 8             | +     | 0    |                |      |
|  |            | 1             | 0     | +    |                |      |
|  |            | 35            | 0     | 0    |                |      |
| Persons without clinical symptoms definitely exposed to whooping cough | 6          | 2             | +     | +    | 2              | 2    |
|  |            | 4             | 0     | 0    |                |      |
| Persons with colds or coughs probably exposed to whooping cough        | 81         | 7             | +     | +    | 14             | 9    |
|  |            | 7             | +     | 0    |                |      |
|  |            | 2             | 0     | +    |                |      |
|  |            | 65            | 0     | 0    |                |      |
| Persons with no illness probably exposed to whooping cough             | 2          | 2             | 0     | 0    | 0              | 0    |
| Persons with colds and no known exposures to whooping cough            | 45         | 2             | +     | +    | 2              | 2    |
|  |            | 43            | 0     | 0    |                |      |
| Persons without illness and no known exposures to whooping cough       | 72         | 3             | +     | +    | 3              | 4    |
|  |            | 1             | 0     | +    |                |      |
|  |            | 68            | 0     | 0    |                |      |
| Miscellaneous sera for pertussis diagnosis                             | 11         | 2             | +     | +    | 6              | 3    |
|  |            | 1             | 0     | +    |                |      |
|  |            | 4             | +     | 0    |                |      |
|  |            | 4             | 0     | 0    |                |      |
| Total no. of sera  | 342        |               |       |      | 87             | 64   |
| Total positive sera  | 92         |               |       |      |                |      |

\*Each specimen was pooled from a group of mice vaccinated with the same antigen.

†Each serum represents a group of normal mice.

TABLE II  
PERTUSSIS SLIDE VERSUS TUBE AGGLUTINATION  
(Comparison of Agglutination Titers on the Slide and in the Tube)

| NO. OF SERA | AGGLUTINATION TITER |                 | REMARKS   |
|-------------|---------------------|-----------------|---|
|             | SLIDE               | TUBE            |   |
| 11          | 1:300-1:1,024       | 1:1,600-1:9,600 | The agglutination titer is given in terms of the highest dilution that showed definite clumps |
| 6           | 1:64 -1:256         | 1:300 -1:2,400  |   |
| 13          | 1:16 -1:40          | 1:50 -1:6,400   |   |
| 10          | 1:5 -1:10           | 1:25 -1:400     |   |
| 10          | 1:2 -1:4            | 1:10 -1:75      |   |
| 9           | 1:1                 | 1:10 -1:150     |   |
| 14          | 1:2 -1:8            | 0               |   |
| 14          | 1:1                 | 0               |   |
| 5           | 0                   | 1:5 -1:37.5     |   |
|             |                     |                 |   |
|             |                     |                 |   |
|             |                     |                 |   |

Total number positive, 92 sera; positive on slide and tube agglutination, 59 sera; positive on slide only, 28 sera; positive on tube only, 5 sera.



centrated form at 8 to 10° C. On the day of the test the antigen was diluted to contain approximately 2,500 million bacilli per milliliter.

*The Test.*—The sera were tested in 1:10 to 1:1,000 dilution or higher if necessary. To 0.1 ml. of the serum dilutions, 0.9 ml. of the antigen was added, and the tests were incubated in a water bath overnight at 52° to 56° C. An antigen control in saline without serum, and an antigen titration with a positive serum were included in each test. Readings were made macroscopically, using a constant light. The titer of the serum was recorded in terms of the highest dilution that showed distinctly visible clumps. For the purpose of comparison with slide agglutination, a serum was recorded positive on tube agglutination when there was distinct agglutination in the 1:10 dilution of the serum. Doubtful or no agglutination in the 1:10 dilution when all the higher dilutions were negative was recorded as negative.

We tested 342 sera for slide and tube agglutination. Among these were sera from persons who were vaccinated against pertussis; from groups of mice that were immunized with various pertussis antigens; from persons with clinical pertussis; from persons with colds or coughs, who were exposed to pertussis; from persons without illness some of whom had been exposed to pertussis; and from groups of normal mice. The results are summarized in Tables I and II.

Table I shows that more positive results were obtained on slide than on tube agglutination with sera of persons who had clinical pertussis and those who were suspected of having pertussis, or were exposed to actual cases of this disease. Slide agglutination appears to be specific since no more positive results were obtained on the slide than on tube agglutination among persons who were not exposed to pertussis, although a large number of them had coughs or head colds at the time of the test.

Table II shows that the agglutination titers on the slide and in the tube did not run parallel. On the whole however, all of the sera that had a high titer on tube agglutination also had a fairly high titer on slide agglutination. Slide agglutination proved to be more sensitive than tube agglutination in detecting weakly positive sera as there were 28 sera positive on the slide and negative on tube agglutinations, as compared with 5 sera that gave a low titer of agglutination in the tube and were negative on the slide.

#### CONCLUSION

1. The results obtained in this study have confirmed the findings of Evans and Maitland that more positives are obtained on slide than on tube agglutination in detecting pertussis antibodies.

2. We found that an antigen that was preserved with merthiolate and stored at 8 to 10° C. had given equally good results as a live antigen that was recommended by Evans and Maitland, and it retained its agglutinability during the seven months' period of this study. In all probability it will remain unchanged for a much longer time. This antigen has the advantage over a live antigen in that it is always available and it eliminates the necessity of preparing and standardizing the antigen for each day's test.

3. The results obtained on slide agglutination were as specific as on tube agglutination since no more positives were obtained on the slide than on tube agglutination with sera from persons who had no clinical symptoms of pertussis and no history of exposure to pertussis.

4. Slide agglutination has the advantage over tube agglutination in that the result may be obtained within a few minutes, and with a carefully standardized antigen more positive results may be obtained with weakly positive sera.

5. In the testing of large numbers of sera, slide agglutination would be very helpful in eliminating negative or doubtful sera. The positive sera could then be titrated by tube agglutination if the exact titer of the serum is required.

6. From present indications a positive result on slide agglutination may be taken as diagnostic of pertussis infection, when there was no previous pertussis vaccination and no vaccine therapy. A negative result does not exclude pertussis infection since a fairly large number of clinically typical pertussis patients do not develop agglutinins until late in the disease or until convalescence.

Technical assistance in this study was given by Edna Lewis and Ruth Orange in carrying out the tube agglutination tests.

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# A SIMPLE DEVICE FOR THE PROTECTION OF THERMOSTATS IN WATER BATHS\*

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**I**N MANY electrically operated water baths, the thermostatic control necessary to maintain a constant temperature is exposed to the destructive action of mercury if the thermometer is broken.

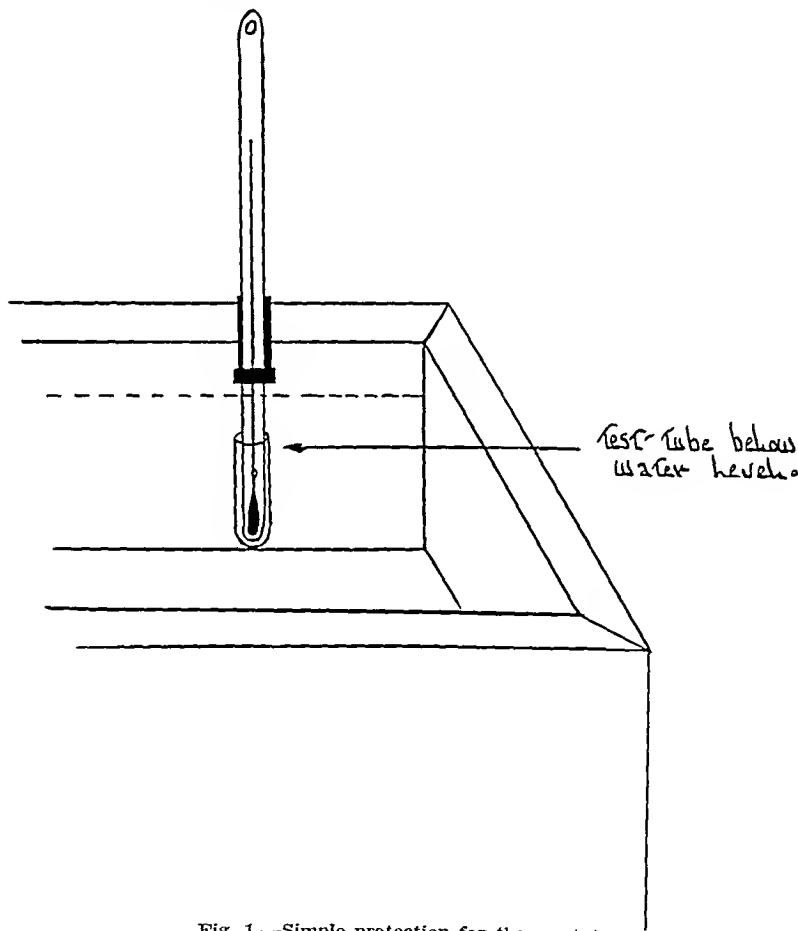


Fig. 1.—Simple protection for thermostats.

Mercury can ruin a water bath beyond repair by attacking the solder of joints in the bath and the metals in the thermostat. This can be avoided by placing a small test tube around the bulb of the thermometer in such a manner that the top of the tube is below the water level. The test tube can be adjusted to fit the type of water bath and thermometer as required. In the event of thermometer breakage, the mercury will fall into the test tube. A simple clamp device that will hold the tube upright can be constructed, depending upon the type of water bath. The mercury therefore will not come in contact with metal connections.

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## THE ANTIFOGGING EFFECT OF SOAP ON EYEGLASSES AND DIAGNOSTIC MIRRORS\*

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TO ANY physician who has had to operate wearing eyeglasses and a face mask, fogging or steaming of the glasses is a real hardship. The mask diverts the warm exhaled air along each side of the nose and causes it to condense on the cooler surfaces of the eyeglass lenses. Fogging results, and this makes it difficult for him to see through the lenses.

Johnson<sup>1</sup> discussed this problem in a short article and contributed a unique solution. He attached a piece of adhesive tape along the top of the mask and pasted this to the skin of the nose. This does not allow the warm air coming from the mouth and nose to be diverted toward the glasses. Other contrivances, such as metallic inserts along the top of the face mask and loosely fitting sacs covering the whole head, have also been tried, but these require special equipment.

The present contribution concerns means of treating the glasses themselves so that the warm air cannot condense on their surfaces. It consists of chalking a few lines across the lens with a piece of soap, and then polishing the glass with soft paper or a handkerchief. A thin coating of soap remains on the surface which repels the condensation of the warm air. The visibility is not decreased by this fine coating.

The method is also applicable to laryngeal mirrors and other diagnostic mirrors that come in contact with warm, moist air. Other methods, such as warming the mirror or coating it with saliva, are in use, but the simplicity of the present plan serves to recommend it. For diagnostic mirrors, I have found the antifogging effect of naphtha soap to be more effective than ordinary white soap.

Realizing that a solution so simple could hardly be original, I referred to a book<sup>2</sup> published by Henley and Company, which contains among its 10,000 formulas, one which prevents the "dimming" of eyeglasses and other glassware. The formula consists of adding a form of soap to glycerin and oil of turpentine.

### SUMMARY

Soap exerts an antifogging action on eyeglasses and diagnostic mirrors.

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## A SIMPLIFIED "LYOPHILE" DESICCATOR FOR SMALL LABORATORIES\*

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A NUMBER of devices have been described<sup>1,2</sup> for the desiccation of biological materials from the frozen state by evaporation of the water under a high vacuum, with subsequent condensation of the vapor in a condenser held at a very low temperature. For this process, the term "lyophile" has been proposed.<sup>1,3</sup> These devices have been intended primarily for the processing of relatively large volumes, and as a consequence have a much greater capacity than is necessary for the handling of occasional small batches of material. Further, these devices are in general of a somewhat complicated design, necessitating the services of a machine shop and skilled workmen in their construction. It is the purpose of this paper to describe a modification of the lyophile apparatus, developed for use in a small laboratory, which possesses certain advantages with regard to convenience, adaptability, simplicity of construction, and inexpensiveness.

Fig. 1 presents a diagram of the apparatus. *A* is the condensing unit, a 1 liter, short neck, pyrex flask, fitted with a 34/45 standard taper ground glass joint and a 3-way stopcock of large bore, *C*. *B*, the connection between the condenser and the vessel containing the material to be dried, *F*, is made of pyrex tubing with an internal diameter of approximately  $\frac{3}{4}$  inch, bent at right angles at each end, and fitted with two 34/45 standard taper ground joints (inner part) as illustrated. The end fitting the condenser is extended beyond the ground joint to within  $1\frac{1}{2}$  inches of the bottom of the flask. *D* is a large metal can holding the freezing mixture of dry ice and 95 per cent alcohol; it in turn is held in a wooden box *E* surrounded by 2 inches of fine sawdust or other suitable insulating material.

In use, a mixture of cracked dry ice and alcohol is placed in the metal container, and the condensing flask is immersed to thoroughly chill it. Five pounds of dry ice will operate the apparatus for six hours or more. The alcohol may be re-used (usually about six times) until it has absorbed enough water from the atmosphere so that ice crystals form in it when the dry ice is added. The material to be dried is frozen around the interior of a suitable container by rotating the container and contents for a few moments in the dry ice-alcohol bath. The rate of drying is dependent in a large measure on the total surface area of the material to be dried, so that the layer of frozen material is made as thin as practical. The apparatus is then assembled as illustrated, the joints being made vacuum tight by means of a suitable sealing compound such as "Lubri-Seal." Evacuation of the apparatus is carried out by means of a Cenco

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Hyvac pump, or its equivalent, attached to the three-way stopcock during the entire period of desiccation. A McCleod gauge is not essential to determine the degree of vacuum. In our experience the pump is operating satisfactorily if the valves make a sharp metallic click as they seat. It has not been found necessary to interpose a trap between the condenser and the pump to prevent water vapor from entering the pump, as suggested by several workers.<sup>2, 3, 5</sup> In this laboratory the oil in the pump is changed every four to six months, and while it contains some water, it does not seem to affect materially the operation of the pump. In this connection, removal of the water and debris from the oil by centrifugation in a Sharples centrifuge renders it again fit for use. When desiccation of the material is complete, the vacuum is broken by turning the three-way cock, and the material is removed from the desiccator.

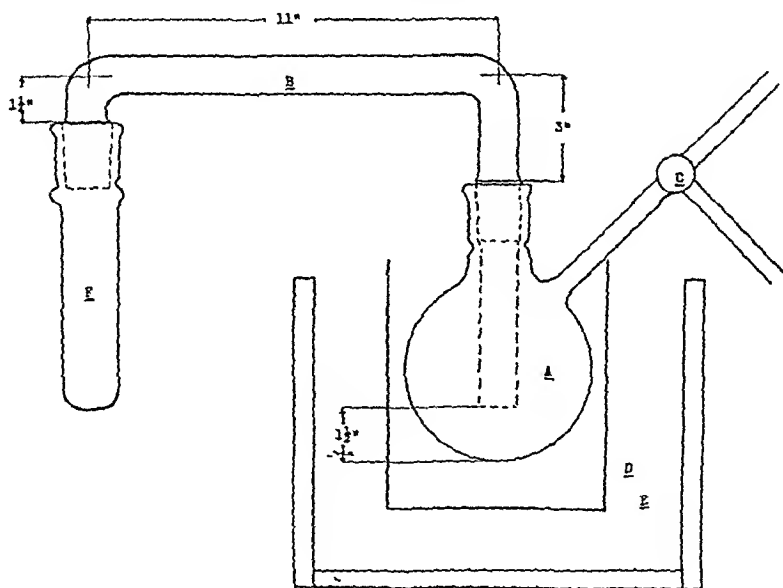


Fig. 1.

By virtue of the standard taper joints it is possible to attach a wide variety of desiccation vessels to the apparatus. For desiccation of small volumes of material, a container made by sealing the open end of a 34/45 standard taper pyrex ground joint (outer part) has proved very satisfactory (Fig. 1, F). The most generally useful desiccation vessel in this laboratory has been a small vacuum desiccator (140 mm. inside diameter) with a ground joint in the lid fitting the pyrex 34/45 ground joint. It is not necessary to have this lid made to special order, since it was found after testing several sizes and makes of desiccators that several are made with a ground joint fitting perfectly with the pyrex standard taper joint. Corning Glass Works also manufacture a large pyrex vacuum desiccator, which is equipped with a 34/45 standard taper joint in the lid. In using either the small or large desiccator, the material to be dried is frozen around the sides of one or more beakers or other suitable containers, placed in the desiccator, and the desiccator is attached to the condensing unit.

Evaporation of the water vapor proceeds at a sufficiently rapid rate so that the material to be dried remains frozen during the entire period of desiccation. As shown by Greaves and Adair,<sup>1</sup> sublimation of water vapor can be materially increased by raising the temperature, taking care, of course, to avoid melting the frozen material. In using a flask as a desiccating vessel, this warming may be accomplished by immersing the flask in a water bath at 30° to 40° C. At such temperatures the material will remain frozen if the pump is operating satisfactorily. The vacuum desiccators may be wrapped with rubber tubing held in place with adhesive tape. After a high vacuum has been attained, warm water may be circulated through this tubing. It is necessary to use a somewhat higher temperature here (40° to 50° C.), since the vacuum within the desiccator insulates the contents to a certain extent. It is impossible to give figures on the rate of drying, since this rate is dependent on so many factors: the material to be dried, surface area of the material, degree of vacuum, temperature of the desiccation vessel, and the like. However, when drying antisera, frozen around the sides of 600 ml. beakers, a rate of about 15 to 20 c.c. per hour was observed.

The chief advantage of this modification of the lyophile apparatus is its versatility. In addition to the desiccation vessels mentioned, there are in use in this laboratory several sizes and shapes of flasks and a pyrex manifold for the desiccation of small amounts of material in tubes with subsequent sealing of the tubes under vacuum. Other advantages of this apparatus are its simplicity and economy. A moderately skilled glass blower should be able to construct it from standard items of pyrex glassware in a few hours, or the Corning Glass Works will manufacture the apparatus to specifications for a reasonable sum. By no means least of the advantages is the ease and simplicity of cleaning and removal of frozen water vapor. It is necessary merely to disconnect the condenser from the connecting tube, thaw under the tap, and empty. This operation may be performed rapidly enough so that the material in the desiccation vessel does not thaw.

This apparatus has been used in this laboratory primarily for the desiccation of partially purified fibrinogen and prothrombin preparations to be used in the study of streptococcal fibrinolysin.<sup>2</sup> The precipitated material, either fibrinogen or prothrombin, is dissolved in a small amount of water, frozen, and dried. It has been found that desiccation in the lyophile apparatus instead of over phosphorous pentoxide, as originally recommended,<sup>7,8</sup> results in greater solubility, less denaturation, and greater stability of these materials. In addition to fibrinogen and prothrombin, materials dried in this apparatus have included partially purified fibrinolysin, bacterial polysaccharides, cultures of various organisms, virus suspensions, plasma and serum, and various antisera. Other possible uses include preservation of complement, typing sera, and soluble antigens.

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## SIMPLE MODIFICATION OF THE HANIKE-GIBBS DROP RECORDER\*

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THE Hanike-Gibbs instrument for recording drops of secretion applies the principle of displacement of an electrolyte solution with constant viscosity and conductivity. The instrument reported by Gibbs<sup>1</sup> in 1927 consists of 5 different parts with a number of connections and does not lend itself easily to experiments in which the rates of a number of different secretions must be measured at the same time. We have therefore modified the instrument, using the same principle.

Fig. 1 is a diagram of the modified drop recorder. The upper part of two glass cylinders *A* and *B* is solidly connected by a glass tube *C*. The first cylinder *A* is empty and connected to the cannulated duct of an organ, the secretion of which is to be measured. The second cylinder *B* is filled with a 10 per cent solution of sodium citrate.† The lower pole of this cylinder has a fine opening *F* through which a drop of the citrate solution is forced when a drop of secretion displaces air in the first cylinder. The falling citrate drop passes between platinum wire *K* and the brass funnel *L*, fixed in a glass tube *G* to which slight constant water suction is applied so that the drop of citrate passes rapidly between the contacts. The drop closes the electric circuit temporarily while touching the two contacts, and the signal magnet is activated. The contacts are connected from binding posts *H* and *J* in series with 115 volts A.C. or D.C. and to a small recording signal magnet. A fuse is introduced into the circuit. Due to the constant suction the amount of current transmitted is small enough to permit the use of an ordinary 6-volt laboratory signal magnet. The instrument can be made in the laboratory at low cost. We have used it for more than two years and found it so reliable, easy to use, and maintain that we feel it would be useful to many other laboratories. The instrument must be protected from radiating heat which will expand the air and citrate solution in the two chambers. The instrument will work for many hours unsupervised.

\*From the Department of Gastro-Intestinal Research, Michael Reese Hospital, Chicago. A preliminary report was published in the *Proc. Am. Physiol. Soc.* p. 214, April, 1941.

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†Lately we have used instead a 3 per cent solution of commercial sulfuric acid, which is cheaper and which keeps the contacts *K* and *L* clean.



Samples of secretion can be removed from *B* at intervals for analysis. The samples are not diluted or mixed with other substances, and they are not exposed to electric sparks and electrolysis as in some other instruments. Refilling can be done swiftly and easily by turning the current off (a switch can be inserted at *J*), opening the clamps at *E* and *D*, and attaching a funnel to the

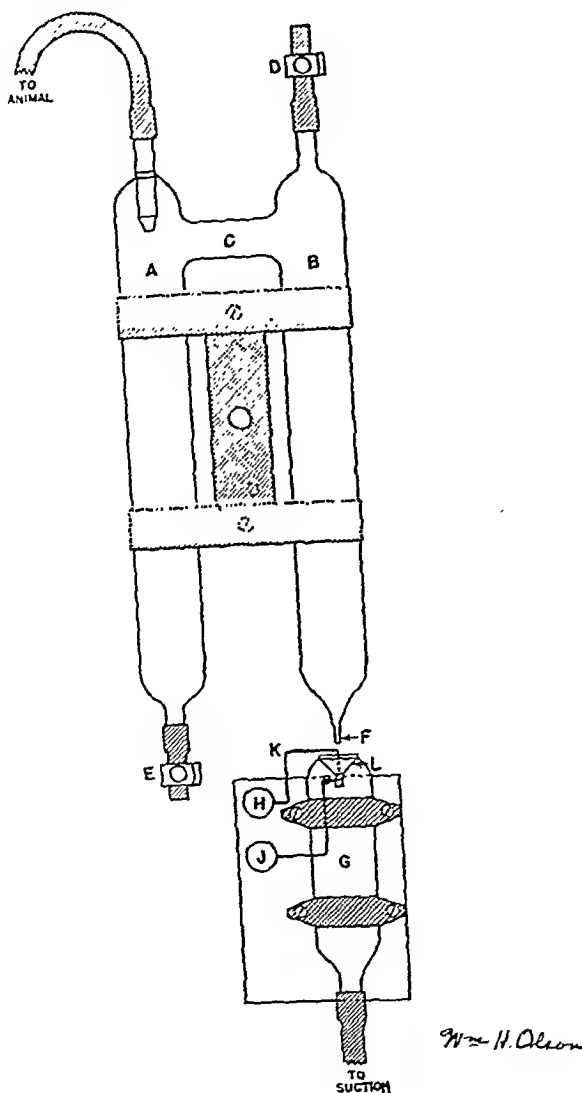


Fig. 1.—Diagram of drop recorder.

rubber tube at *D*. After *B* has been filled with the citrate solution to just below the level of *C*, clamp *D* is closed quickly. During the process of filling, some of the citrate solution escapes through *F* which may be closed during the filling, but we have not found that necessary. It may take then a few minutes before pressure equilibrium is reached in the system. We have found it of advantage to put the drop recorder below the level of the cannulated duct of the animal, because a slight suction on the duct may prevent clogging or, in the case of lymph, clotting.

We have used a set of 4 instruments, as shown in Fig. 2, to record simultaneously salivary, pancreatic, biliary, and lymph secretion.

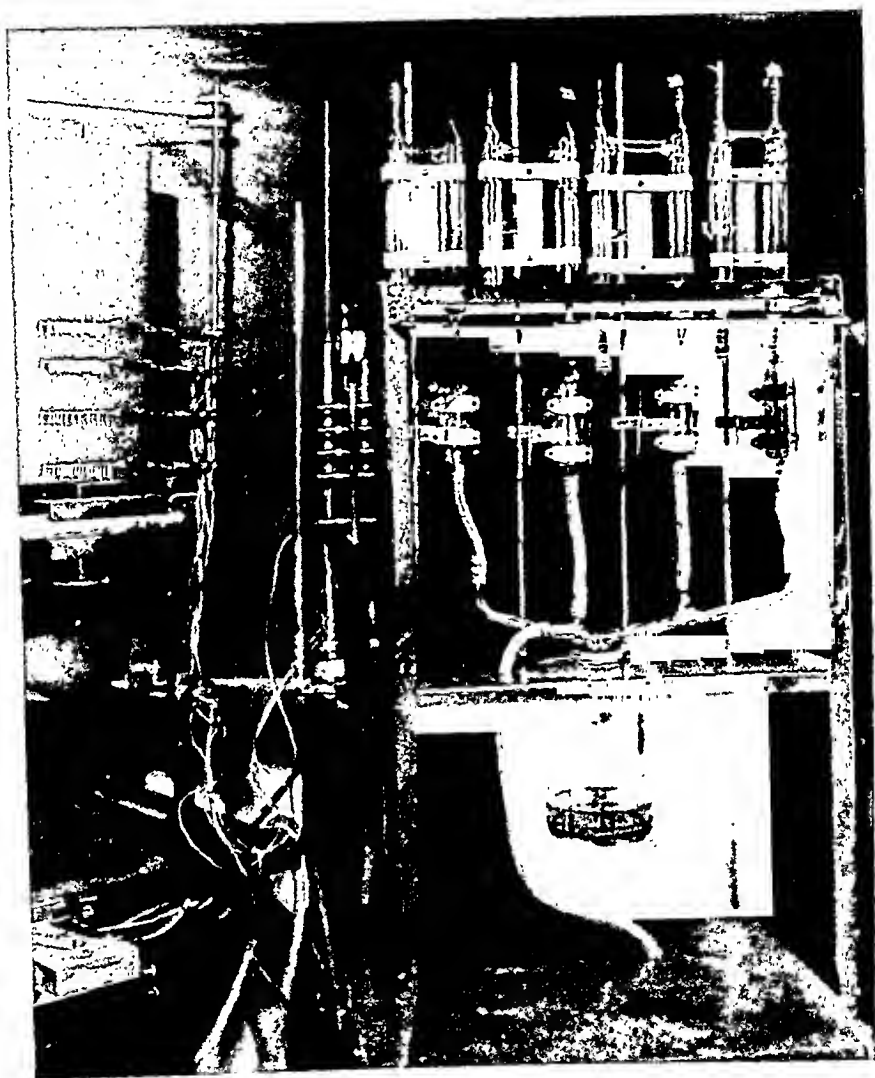


Fig. 2.

#### DISCUSSION

We feel that, when a continuous quantitative record of the volume of a secretion is wanted, the displacement system should be employed in order to obtain more accurate results. For example, in the measuring of the secretion of bile or saliva, the concentrations of salts and of mucins usually change during the experiment. This results in a change in the viscosity of the secretion and in the size of the individual drop. Thus we have often found the size of a drop of bile to double its volume as the experiment progressed. Such uncontrollable changes can be avoided when a solution of constant composition is displaced by the secretion.

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## A SATISFACTORY TECHNIQUE FOR DELAYED TYPING OF THE PNEUMOCOCCUS\*

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WITH the widespread immediate use of the sulfa drugs instead of serum in the treatment of pneumonia, the typing of the pneumococcus seems of little value except for case records. Laboratory directions always state that a sputum is best typed as soon as possible after collection because of the early death of the pneumococcus and the overgrowth of contaminating bacteria. This work is presented to show that this is unnecessary except when "stat" typings are desired.

The suggested technique is based on the following work. Typings were run on more than 50 sputa of known type. Three generous smears were made on each of 6 glass slides and allowed to dry in the air. They were then wrapped in paper and stored, 3 at room temperature and 3 at icebox temperature. After intervals of twenty-four, forty-eight, and seventy-two hours, and one, two, three, and four weeks, each type was tested for the quellung reaction in the ordinary manner. It was found that all slides stored at either temperature for the varying lengths of time typed just as well as the original specimen. A few slides tested after storage for ten months gave good quellung reactions. There was no apparent reduction in the number of swellings or the size of the swollen capsule. Group sera swelled stored preparations as well as the type sera. To check against high temperatures of the tropics several types when dried were stored at incubator temperature (37.5° C.) for one month. These still gave good swellings. All types have been tested except types IX, XI, XXVII, XXVIII, XXXI, and XXXII which were not encountered during the period when the work was being done.

As a result of this work, it is suggested that exudates (with a few exceptions such as spinal fluids requiring centrifugation) immediately upon collection be smeared entirely across or at three locations on each of at least four clean slides and allowed to dry in the air. These may then be typed at one's convenience in the usual manner.

Since this seems to prove that delayed typings are possible, several advantages can be observed in the use of dried slides for typings:

1. Dry fields are easier to examine microscopically since there is less motion present than in a moist smear.
2. Drying on a slide keeps the organisms from deteriorating and contaminating bacteria from overgrowing and destroying the pneumococci.

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3. Dried slides can easily be mailed great distances for typing, and variance in time would not alter the results.

4. Smears from the pharynx, especially in children, can be made immediately and typed easily by this method without the risk of the swab's drying before it can be typed.

5. The most important advantage from the technician's standpoint is the lack of the necessity of "stat" work.

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## TRYPSIN AS A DIGESTANT OF SPUTUM AND OTHER BODY FLUIDS PRELIMINARY TO EXAMINATION FOR ACID-FAST BACILLI\*

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A NUMBER of methods have been used for the concentration of sputum preliminary to examination for acid-fast bacilli. Some of these fail to concentrate the organisms adequately; others are time consuming; and some require special apparatus and delicate technique. Since 1933 we have used an aqueous solution of trypsin to digest such specimens as sputum, stomach washings, pleural and abdominal fluids, urine that contained mucus, and pus from abscesses, with satisfactory results.

### METHOD

A 0.5 per cent trypsin solution is made by adding powdered trypsin† 1:250 (Difco) to sterile water. Mix by shaking, and add 0.7 per cent N/1 sodium hydroxide solution, which gives approximately pH 8.5. Since trypsin acts over a wide range of hydrogen-ion concentration, it is not absolutely essential that the sodium hydroxide be added, but the trypsin is most active at pH 8.0 to 8.5. The trypsin solution should be kept in the refrigerator or, better, filtered through a Seitz filter if it is not used shortly after it is prepared.

1. An equal quantity of the trypsin solution, or more if the specimen is very thick, is added to the specimen in a bottle or large test tube, and mixed thoroughly, an applicator stick or glass rod being useful for this purpose.

2. The specimen is then incubated at 37° C. for thirty minutes, or until the mucus is thoroughly digested, which can be determined by drawing out the applicator stick or by the sedimentation of the cells. Stirring after ten to fifteen minutes' incubation may hasten the digestion.

3. After digestion is complete, the specimen is centrifuged at 3,000 r.p.m. for five minutes, or at 1,500 r.p.m. for ten minutes.

4. A loopful of the sediment is smeared on a glass slide, fixed as usual, and stained by the Ziehl-Neelsen method. If the smear is thin, methylene blue is used as a counterstain; if it is thick, 1 per cent aqueous picric acid is used. The sediment is also satisfactory for examination by the fluorescence method.

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†Difco Laboratories, Inc., Detroit, Mich.

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## EXPERIMENTAL DATA

In order to compare the effectiveness of concentration of acid-fast bacilli in sputum by the trypsin method with the flocculation method of Hanks et al.,<sup>1</sup> a series of specimens was examined by both methods. Preliminary to digestion the specimens were heated in an Arnold sterilizer for thirty minutes and cooled (this is done routinely in our laboratory as a safety measure unless the specimen is to be used for culture or guinea pig inoculation). The specimens were then divided as evenly as possible (we used as much as 5 c.c. if the size of the specimen permitted) and put into 6 inch by  $\frac{3}{4}$  inch test tubes. The procedure of Hanks et al.<sup>1</sup> for the concentration of tubercle bacilli and the trypsin method as outlined above were followed. Smears were made in a uniform manner, a 4 mm. loopful of the sediment being spread over an area 1.5 cm. by 3.5 cm., and fixed and stained by the Ziehl-Neelsen method. The acid-fast bacilli in 200 fields were counted, the number of fields being recorded as the bacilli were counted. Since the finding of a clump of bacilli does not increase the possibilities of finding the organism, each clump was counted as one.

Of 100 specimens of sputum which were positive by one or both methods, 14 per cent were positive by the trypsin method that were negative by the flocculation technique and 3 per cent were negative by the trypsin method which were positive by the flocculation technique. In 5 per cent of the specimens the same number of bacilli were found by both methods. In the remaining 95 specimens more acid-fast bacilli were found in 71.5 per cent of the trypsin-digested specimens, and in 30 per cent of these at least twice as many acid-fast bacilli were found in 200 fields by the trypsin technique as by the flocculation method. More acid-fast bacilli were found in 28.4 per cent of the specimens by the flocculation method, and 7 per cent of these had at least twice as many acid-fast bacilli as were found by the trypsin method.

## DISCUSSION

As the results show, 11 per cent more specimens were positive by the trypsin method than by the flocculation method. Also more acid-fast bacilli were found in 71.5 per cent of the trypsin-digested specimens in contrast to 28.4 per cent of the specimens which showed more acid-fast bacilli in the portion of the specimens concentrated by the flocculation method. This indicates that a specimen might be found positive by both methods, but the bacilli would probably be found in less time in smears made from the trypsin-digested specimen. This is indicated further by the fact that 30 per cent of the smears made from the trypsin-digested specimen showed at least twice as many bacilli as the same specimen concentrated by the flocculation technique, while, of those smears which gave a higher count by the flocculation method, only 7 per cent had at least twice as many of the bacilli as were found by the trypsin method.

Not only is there a reduction in the time required for examination of smears over that required by some other methods, but the time necessary to carry out the procedure of digestion and concentration is reduced to a minimum. The steps of the procedure are few and simple. No delicate or involved technique is necessary, and no special apparatus is required. Although sputa vary considerably in hydrogen-ion concentration (from pH 5.0 to pH 7.5), it is not necessary to adjust each specimen to a certain hydrogen-ion concentration since trypsin is active over a wide range.

Sedimentation always seems to be very complete after thorough digestion of the mucus. The specific gravity is low (around 1.006 to 1.011), and the surface tension is such that it does not interfere with complete sedimentation. Recentrifugation of the supernatant fluid from a few positive specimens gave very little or no sediment and no positive smears.

Digestion of sputum which has been heated proceeds faster than that of unheated sputum if it contains considerable mucus. After heating, specimens may contain lumps of coagulated material, but these are soft and easily broken by means of an applicator stick or glass rod.

Considerable sediment is obtained from trypsin-digested specimens as the other organisms and body cells present are not digested. This we consider an advantage as they give a background which enables the technician to keep the field in focus more easily. Also any other organisms that may be of clinical importance, such as those of Vincent's infection and fungi, may be seen. If the smear is thick, however, picric acid is preferred as a counterstain, for methylene blue may stain so heavily that the acid-fast bacilli cannot be seen. If picric acid is used for the counter stain, a smear can be stained by another method for other organisms. The sediment is usually creamy in consistency and spreads easily, and the smears stick well to the slide.

The presence of phenol in specimens of sputum does not interfere with digestion by trypsin. Specimens in which unknown amounts of phenol were present have been digested with trypsin solution with no difficulty. To one specimen an equal amount of 5 per cent phenol was added and to that mixture an equal amount of trypsin, giving 1.25 per cent of phenol. No inhibition of the enzymatic action was observed; on the contrary, it seemed to be stimulated. It might be stated that trypsin has been used for the digestion of sputum specimens by the Indiana State Board of Health for the past three years, with satisfaction, all of the specimens being collected in bottles which are from one-third to one-half full of 5 per cent phenol when issued from the laboratory.

For the past eight years we have also used trypsin to digest specimens preliminary to culturing or guinea pig inoculation. Acid-fast bacilli treated as long as twenty-four hours with trypsin solutions are viable and will infect a guinea pig. After digestion of the specimen with trypsin the sediment is treated with oxalic acid to kill the other organisms present according to Corper's method<sup>2</sup> for culture, or prepared for guinea pig inoculation.

The trypsin powder is not sterile, and bacteria and higher fungi will grow in a solution of it, even at low temperatures. As these might prove a source of error, sterilization by filtering through a Seitz filter is recommended. However, we have found that unfiltered trypsin solution, if made with aseptic precautions and kept in the refrigerator, will remain in good condition for a few days, but one must be alert to the possibilities of error when using it. Also, if the solution is used immediately after preparation for the digestion of specimens for smears, it is not necessary to filter it. To digest specimens for culturing or guinea pig inoculation, sterile trypsin solution should be used.

Trypsin powder is relatively inexpensive. The amount needed for the digestion of a specimen costs less than one cent.

## SUMMARY

A method of digesting sputum and other body fluids with trypsin preliminary to microscopic examination, enturing and guinea pig inoculation, has been presented.

This method is considered to be practical for it is simpler in procedure than other satisfactory methods and requires less of the technician's time.

In a comparative study of this method and the flocculation method of Hanks et al.,<sup>1</sup> of 100 positive specimens, 11 per cent more positives were found by the trypsin method. In 71.5 per cent of the smears made from the trypsin-digested portion of the specimens, more acid-fast bacilli were found than in smears made from the flocculation concentrate, and in 30 per cent of these at least twice as many bacilli were found. In contrast to these results, only 28.4 per cent of the smears made from the flocculation concentrate showed more bacilli than those made from the trypsin-digested portion of the specimen, and, of these, only 7 per cent showed twice as many or more acid-fast bacilli.

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AN IMPROVISED INVERTED MICROSCOPE<sup>\*1</sup>

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THE inverted microscope has been employed in photomicrography,<sup>2,3</sup> in tissue culture,<sup>4</sup> and in micrurgy.<sup>5</sup> Special advantages accrue from its employment in these various fields, and in the field of micrurgy these advantages have been sufficient to warrant the appearance of a commercial instrument.<sup>5</sup>

A description is herein given of an improvised inverted microscope which possesses the advantages of being readily available and inexpensive, for, except for two small prisms, the instrument is assembled from (1) microscope parts commonly available in the laboratory and (2) a few special parts which can easily be fabricated. The microscope parts used in the assembly suffer no mutilation. The instrument is designed especially for employment in conjunction with the Taylor micromanipulator,<sup>6</sup> but no difficulty should attach to its alteration for employment in conjunction with the Chambers micromanipulator.<sup>5</sup>

Fig. 1 presents the essentials of the arrangement. The arm (A) and body tube (B-T) of a standard microscope are affixed to a cast-iron base (B). A prism-chamber (p-c) replaces the nosepiece, and supports the three standard

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objective lenses. A sturdy upright (*U*), mounted on the base (*B*), supports the condenser (*C*) and light source (*L*). A mechanical stage (*S*) is fixed to the base (*B*) and serves in turn as base for a thin upright (*u*) which supports, between the condenser (*C*) and the objective, a coverslip which serves as a "stage" (*s*).

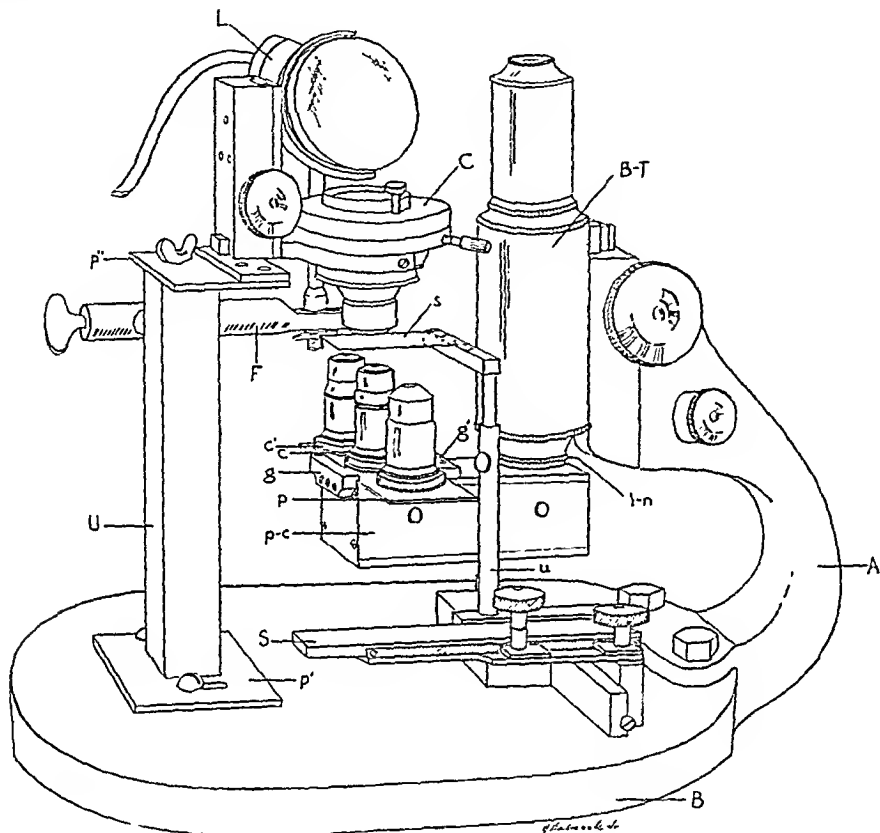


Fig. 1.

The following details concerning the various parts of the instrument are pertinent (see Figs. 1 and 2) :

1. The arm (*A*) and body tube (*B-T*) are taken from a model 66 Spencer microscope.
2. The prism-chamber (*p-c*) is constructed of  $\frac{1}{8}$  inch sheet aluminum. It is  $3\frac{1}{4}$  inches long, and its internal cross section measures 1 square inch. It is screwed into the body tube (*B-T*) by means of a threaded collar (*tc*), and is locked in proper position by means of a lock-nut (*l-n*). Two  $\frac{3}{8}$  inch holes are drilled in the roof of the prism-chamber (*p-c*), one concentric with the body tube (*B-T*) and the other concentric with the objective. Contained within the prism-chamber (*p-c*) are two right-angle prisms, as indicated in Fig. 2; these fit snugly into the chamber (*p-c*), and are held in position by means of screws (*x*) projecting through the floor of the chamber (*p-c*) and by plates which close its ends. The three objectives are mounted on a brass plate (*p*), each threaded into a collar (*c*), and this plate (*p*) is movable in a line perpendicular to the length of the prism-chamber (*p-c*). The plate (*p*) moves between two guides (*g, g'*).

3. A nicely-machined band (*c'*) fits snugly about the collar (*c*) into which the objective is threaded and can be fixed by means of a set-screw. Each band (*c'*) bears a small ma-



clined depression (not shown) which, when the desired objective is moved to center position, receives a spring-backed pin (not shown) projecting forward from the rear guide ( $g'$ ). By means of these adjustable bands ( $e'$ ), the objectives may be precisely centered.

4. The upright ( $U$ ) which supports the condenser ( $C$ ) and light source ( $L$ ) is a  $5\frac{1}{2}$  inch length of  $\frac{3}{4}$  inch square brass rod. A brass plate ( $p'$ ) fixed to its lower end serves for attachment to the base ( $B$ ); this plate ( $p'$ ) has slotted screw-holes which allow for adjustment of position. A brass plate ( $p''$ ), fixed to its upper end, supports the condenser ( $C$ ); this plate ( $p''$ ) is attached to the upright ( $U$ ) by means of a centered screw, so that the condenser ( $C$ ) can be swung aside readily.

5. The condenser ( $C$ ) is an achromatic piece, of N.A. 1.3, as supplied for Model 3 Spencer research microscope. The entire assembly, including rack and pinion, is employed.

6. The light source ( $L$ ) is an 18-volt toy train lamp. It is energized with 110-volt A.C. current, a variable rheostat being inserted in the line. The lamp housing is an objective case, in one end of which a frosted glass is mounted. This housing is supported on the upright ( $U$ ) by means of an adjustable framework ( $F$ ).

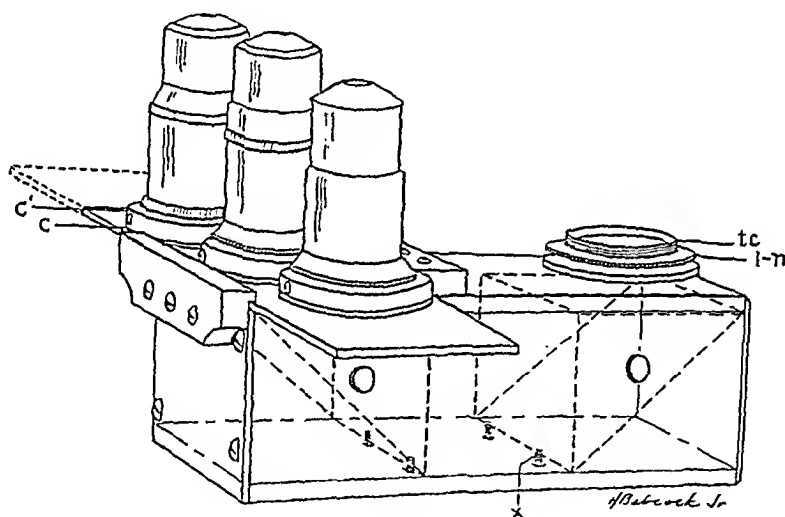


Fig. 2.

7. A Spencer attachable mechanical stage ( $S$ ) is fixed to the base ( $B$ ), slightly to the front and left of the arm ( $A$ ). Its attachment is at right angles to its usual attachment, so that the movement which is normally lateral is here the front-back movement. Rising vertically from the front-back movement is the  $\frac{5}{16}$  inch circular upright ( $u$ ), provided with a centered extension in the form of a  $\frac{3}{16}$  inch rod. A horizontal bar projects centrally from this extension, and supports the coverslip "stage" ( $s$ ).

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# CHEMICAL

## DESICCATED PLASMA FOR NATIONAL DEFENSE\*

### MASS PRODUCTION METHODS BASED ON THE ADTEVAC PROCESS

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THE need of plasma in national defense is too urgent for prolonged consideration of theories and speculations. Extensive clinical results and proved production methods of large capacity are required to answer the following questions:

1. In what form is plasma most suitable for defense needs and military use?
2. Should plasma be administered in hypertonic, normal, or diluted form?
3. By what methods can mass production be achieved?

It is the purpose of this paper to answer these questions in the light of the experience of the desiccated plasma service of the William Buchanan Blood, Plasma, and Serum Center of Baylor University. This service during the past twenty-one months† has separated the plasma from 1,191,000 c.c. of blood, and desiccated plasma has been made available in Dallas and vicinity to the same extent as ordinary intravenous fluids. As a result, the value of this type of plasma used in hypertonic solution has been proved in extensive clinical trial.

On the basis of this experience the following answers to the three questions on use and preparation of plasma are given as follows:

1. For military as well as civilian use plasma desiccated from the frozen state is the ideal type of plasma preparation.

2. It should be used in hypertonic form (four times concentrated) to realize maximum advantages.

3. Mass production can be achieved with complete safety by the following principles: (a) Simplified collection, (b) Pooling whole bloods, (c) Separation of plasma by the continuous separator of the De Laval type, (d) Seitz filtration, (e) Bulk desiccation by the adtevac process, (f) Granulation and packaging of maximum quantity in minimum space in ordinary vaccine vials by a sterile mechanical dispenser, (g) Use of sterile room for open or semi-open phases of above methods, and (h) Bacteriologic control of all steps.

#### ADVANTAGES OF DESICCATED PLASMA

Desiccation from the frozen state offers advantages in the storage, transportation, and use of plasma that can scarcely be challenged. First, quality

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must be considered. The remarkable ability of this method of desiccation to preserve proteins and all biologic properties without change has been pointed out by workers in this field.<sup>1</sup>

Preservation of the proteins without deterioration is essential to assure solubility and to eliminate the possibility of protein denaturation as a cause of reactions. The excellent preservation of proteins by desiccation from the frozen state (sublimation desiccation) has been conclusively demonstrated by the electrophoretic studies of Seudder<sup>2</sup> and by our own clinical results.<sup>3-6</sup> For military use solubility should be measured in seconds rather than minutes, so that resolution may be done just prior to administration whether it be on the field, in the collecting station, in the aerial ambulance, or under whatever conditions encountered in peace or war.

Such rapid solubility has been consistently maintained in the Baylor service and has proved to be one of the chief advantages of the desiccated form over our earlier method of storing concentrated plasma in the frozen state. Solubilities average from thirty to 120 seconds. In actual treatment of shock, 25 Gm. of plasma have been dissolved in 90 c.c. of water and administered intravenously, all within 180 seconds. These rapid solubilities are maintained during storage, apparently depending on the very low moisture contents of 1 per cent and less, attainable by this type of desiccation.

Another advantage of dry plasma which has not been properly appreciated and utilized is the fact that it will not support bacterial growth. In this respect the plasma may be considered as any inert chemical powder. This property is of tremendous advantage for safety in use, for storage, and for mass production.

Contrary to the statement of Blalock and Mason,<sup>7</sup> a very definite advantage of reduced bulk is realized for desiccated plasma when packaged as described herein. For example, we placed amounts of plasma and water in two 60 c.c. vials, which is equivalent to 200 c.c. of normal plasma. This becomes a real advantage when the equipment for administration is included as discussed below.

The use of desiccated plasma has been considered expensive<sup>8</sup> and freezing proposed as an alternative method of preservation. From earlier extensive experience we have found that the routine preservation of plasma by freezing had many disadvantages which were eliminated by preservation in dry form. Low temperature refrigeration is costly and may well exceed the small cost of desiccation. When the extra safety and elimination of waste through spoiling or outdating are considered, desiccated plasma becomes definitely more economical. Furthermore, the problem of fibrin precipitation during storage of liquid plasma or upon thawing of frozen plasma is largely eliminated by desiccation. In the dry form fibrin precipitation is impossible and after resolution there is no increased tendency toward fibrin formation.

#### HYPERTONIC PLASMA, THE IDEAL FORM FOR ADMINISTRATION

One of the greatest advantages of desiccated plasma is that it provides the most logical way to make concentrated plasma solution. The ideal form in which to give plasma, particularly for the treatment of shock, is the four times concentrated plasma immediately after redissolving in pyrogen-free water.

Numerous advantages, both technical and therapeutic, realized by giving this hypertonic plasma are (1) increased simplicity and speed of administration with a glass syringe replacing more complicated apparatus, (2) elimination of tubing and the work of preparing it, (3) reduction of bulk and weight through decrease in water carried, and (4) greater therapeutic effectiveness with speedier response.

The technical advantages of administration of plasma in the hypertonic form as listed are obvious. Simplicity, safety, and speed attained by this technique are always important in the treatment of shock but are doubly so in military use. If plasma can be given in a shell hole, this is the way to do it. With a 50 or 100 c.c. glass syringe the hypertonic (four times concentrated) solution can be given as easily as 50 per cent glucose.

Technical advantages are significant only if therapeutic effectiveness is assured. Bond and Wright<sup>9</sup> first demonstrated that concentrated serum was effective in the treatment of shock in dogs. Best and Solandt<sup>10</sup> in a larger series also showed concentrated serum to be effective in the treatment of traumatic and posthemorrhagic shock in dogs, but later Magladery, Solandt, and Best<sup>11</sup> denied any advantage of concentrated over whole serum. Clinical reports by Ravdin, Stengel, and Prushankin,<sup>12</sup> Levinson, Rubovits, and Neeheles,<sup>13</sup> and Strumia, Wagner, and Monaghan<sup>14</sup> have dealt with isolated cases of hypoproteinemia treated with concentrated serum or plasma, but its routine use as a superior therapeutic weapon in the treatment of shock was first reported in 1940.<sup>3</sup>

No large clinical series had been reported until the papers of Brown and Mollison,<sup>15</sup> and Hill, Muirhead, Ashworth, and Tigertt,<sup>5</sup> although preliminary summaries of comparatively large numbers of cases were given in our earlier papers. However, sublimation desiccation of plasma and its use in hypertonic form with emphasis on shock was practiced by the Baylor Plasma Service from its inception in August, 1939.

The advantages which we had at first assumed on theoretical grounds have since been brought out in extensive clinical trial. In Baylor Hospital alone concentrated plasma has been given to 276 patients. Of this number, 93 closely followed cases of shock are summarized and the evidence for hypertonic plasma presented in another paper.<sup>16</sup> The results have confirmed our previously stated thesis<sup>3, 5</sup> that the use of concentrated plasma is the ideal treatment of shock. The shock cycle of Moon<sup>17</sup> is quickly broken by a rapid increase of blood volume due to withdrawal of interstitial water. This constitutes a physiologic correction of the pathologic change of shock. Due to the speed of administration, this correction is effected in the shortest possible time. In addition, the extremely rapid increase in blood pressure of the average case strongly suggests a direct stimulation of the vascular system with reduction of vascular capacity.

In posthemorrhagic shock where red cells have not reached critical levels of oxygen carrying capacity, hypertonic plasma is superior to blood in its rapidity of action and instant availability. Where critical red cell levels have been reached, concentrated plasma is of utmost value in combating shock during the period of preparation and giving of blood. For a discussion of this important question reference should be made to an earlier paper.<sup>5</sup>

In answering the theoretical objection that dehydration would contraindicate the use of intravenous hypertonic solution, it is significant that we have found this type of therapy completely effective in the presence of all ordinary grades of dehydration seen in hospital practice. This is in accord with the well-known fact that interstitial water is easily mobilized and large in amount. According to Gamble,<sup>18</sup> this water makes up 15 per cent of the body weight and comprises a very effective reserve for the more important plasma volume. Of course severe dehydration approaching loss of 10 per cent of body weight would appear to be a definite contraindication for hypertonic plasma.

#### MASS PRODUCTION METHODS

The attainment of large volume production on our service has resulted not only from the adoption of new techniques but also from discarding two methods heretofore considered most essential<sup>19</sup>; namely, a closed type of centrifugation, and desiccation in original containers. These two requirements were abandoned because they constituted the chief obstacles to volume production. A partially open method such as described below has been proved perfectly safe in use with the observance of certain essential principles and checks.

First, it can be taken for granted that all precautions of careful technique and adequate bacterial culture control must be observed in any system. Second, conditions for bacterial growth must be completely eliminated following separation of plasma. If it must be stored before drying, it is kept frozen, but better yet is a balanced system where desiccation closely follows separation. In either event we have always employed Seitz bacterial filtration immediately prior to desiccation as an additional safeguard not only against bacteria but also possible pyrogenic substances. An important point in this connection is the use of filter-cel,\* an infusorial earth preparation. This material is best introduced into some part of the system, such as the prefilter reservoir. It is sterilized with the equipment; when the plasma passes through the system, it is mixed with the filter-cel, and an even deposit forms over the surface of the Seitz disc. This coating greatly increases the capacity of the filter. After desiccation, bacterial growth is not supported and dryness is the greatest plasma safety factor. It can be handled in bulk with the same confidence as any other chemical or powder inert to bacterial growth, and it can be measured and placed in final containers with the same ease. It should, of course, be used immediately upon converting again into liquid form. Proper choice of unit sizes eliminates any need for brief storage as a liquid. Yet even here a safeguard is afforded by use in concentrated solution since this form is bacteriostatic to a large degree to all common contaminants.

It is obvious that the period of storage of liquid blood is the only break in the use of these principles. Where such preservation is essential to blood banking, safety can be attained by a completely closed collection system and a short period of storage. In our own service with 400 bleedings per month it has been feasible to keep blood only four to five days before separation of plasma. Of course, in large scale bleeding for national defense no storage would be necessary if the separator methods herein described were adopted for the immediate separation of plasma.

\*A product of Johns-Manville Sales Corporation, New York, N. Y.

These principles have been worked out during the past two years in actual large scale use and have proved to be safe by the fact that no serious harm has resulted from a single dose, and a febrile reaction rate of 0.74 per cent has been attained in 694 administrations at Baylor Hospital. Furthermore, no reactions have been reported from extensive outside use.

The actual production methods, as shown in the American Medical Association Scientific Exhibit,<sup>20</sup> start with the bleeding technique. In our blood bank we have used a vacuum bottle collection technique. A rubber tube with two observation tubes and needles connected has been used. Vacuum is held during venipuncture by clamping a hemostat on the tube. Collapse of the tubing has in no way impeded withdrawal of blood. Our special mechanical shaker has been replaced by the simple expedient, now commonly employed, of inverting the vacuum bottle during collection. By this method the blood is drawn directly into the citrate solution, resulting in immediate mixing.

In bleeding for national defense we have suggested that defibrination might be preferable. Similar types could be bled into large chilled containers and defibrinated by a rotary paddle. The fibrin could be removed by a centrifugal strainer, and the bloods quickly pooled just before separation. Such methods have been standard in animal serum plants and should be considered seriously. Certainly the small hemoglobin content resulting from such methods must not be considered objectionable. We have given hemoglobin solution intravenously clinically up to 21 Gm. within fifteen minutes without reaction.

The second step of our methods is plasma separation. Two De Laval E-19 separators have been operated in cascade, the first unit separating and the second clarifying the plasma. With this smallest available motor-driven model, we have separated 25 liters of pooled blood in thirty minutes, a rate of 50 liters per hour. A true plasma yield of 50 per cent was obtained consistently. Of course, such capacities at high yields cannot be approached by conventional bottle centrifuges or, in fact, by any other methods. Furthermore, separators are available with capacities up to 1,000 liters per hour. Labor costs are reduced, and such large capacities with efficient yields make this method the obvious choice for mass separation. These conclusions are based on the separation of 778,280 c.c. of human blood by the continuous separator principle in four months. The pooling of whole blood prior to separation employed in our service since January, 1940, has many advantages. Typing is unnecessary, and large volumes are more easily handled. Moreover, agglutinins are completely removed, and the resultant agglutination of the corpuscles by forming larger particles aids in efficient separation and higher yields. Proportioning different types of blood or letting the pool stand as reported by Edwards, Kay, and Davie<sup>21</sup> is unnecessary, particularly with the large pools of 25 to 100 bottles which constitute part of our separation routine. We believe this removal of agglutinins resulting from pooling of bloods, as contrasted with the suppression resulting from pooling plasma of different types, is of the utmost importance. For example, we have never found it necessary to restrict the giving of whole blood after administration of large doses of our concentrated agglutinin-free plasma although Mahoney et al.<sup>22</sup> indicate that caution is necessary in the giving of blood following pooled plasma.

Disadvantages claimed for this method<sup>23</sup> have not been encountered during the eighteen months we have used it as a routine. First, contrary to these claims, high rates of hemolysis have not been found. By the method of Bing and Baker,<sup>24</sup> hemoglobin has varied between 20 and 75 mg. per 100 c.c. of unconcentrated plasma. The hemoglobin concentration has depended mainly on the age of the blood. We have discussed this question of hemoglobin elsewhere<sup>25</sup> and believe that it is of no significance whatever in the causation of reaction or other deleterious effects.<sup>25</sup> Indeed, the successful intravenous use of hemoglobin solution as a form of therapy reduces to an absurdity objections based on hemoglobin content.

Second, the method is partially open although we have redesigned covers and spouts to minimize this. Nevertheless, positive cultures were obtained on only three occasions, when the air was dusty during a two-day period from tearing down a nearby plaster wall. Since then, no contamination has been encountered, but, as an additional precaution, the air in our centrifuge room is electrostatically filtered.

Since the separation of plasma is immediately followed by Seitz filtration and desiccation, or freezing, filtration, and desiccation, bacterial contamination is no problem. We regard filtration of this type to be a safety factor of utmost importance as a final check against bacteria and pyrogenic substances. Since we have filtered the plasma from over 1,000,000 c.c. of blood, we cannot agree with Strumia and McGraw<sup>8</sup> that plasma filtration by this method is sufficiently inconvenient to be a valid objection to its use. Of course, precipitation of fibrin occurs, but it need not clog the filter if a filter of proper size is used and filter-aid added to the plasma to adsorb particulate matter, such as fat and fibrin. The decrease of fibrinogen content by filtration seems to be of no clinical importance.

After filtration, the plasma is delivered from the collector into the large processing ampoules by a bell-protected tip. Desiccation from the frozen state in bulk by the adtevac process<sup>3</sup> is then performed. This method utilizes the well-known fact that adsorbents, such as silica gel, have enormous capacities to take up water vapor. For efficient use in sublimation desiccation in vacuo, however, control of adsorbent vapor pressure and maintenance of capacity were required. These requirements, which had previously prevented the successful use of adsorption, were solved by the adtevac process. With maximum loads plasma moisture contents below the 1 per cent level set by Flosdorf and Mudd<sup>1</sup> were reached. With smaller loads moisture contents as low as 0.03 per cent as determined by the method of Flosdorf and Webster<sup>26</sup> were reached.

The chief characteristics of the process which make it ideal for large volume plasma desiccation are as follows:

1. It is not easily overloaded by initial rush of vapor in early phase.
2. Rapid spontaneous freezing eliminates expensive and troublesome pre-freezing.
3. Temporary interruptions of electric current do not interfere with drying. Thawing and spoiling of plasma are avoided.
4. Extreme rapidity of freezing and positive maintenance of plasma in frozen state give high quality product with unusual solubility.

5. Reliability is assured by use of standard equipment such as vacuum pump and ordinary small refrigeration unit for machine parts.

6. There is economy of operation.

7. The process is well adapted to any size capacity required.

That desiccation from the frozen state need not be expensive has been amply demonstrated in the use of the adtervac process. Inefficient methods of separating and handling plasma on a large scale can easily increase labor cost more than the small power costs required for drying.

Transference of plasma from bulk-processing containers to final units is easily accomplished by a stainless metal dispenser that can be sterilized as a unit in the autoclave. With ampoules such as we have used, a closed system during transference can be had by using a compression joint and a flexible bellows or tube. A rotary-driven worm used in our first model granulated and forced the plasma through a small metal tip into the sterile vial standing on the pan of a torsion balance. The delivery tip was protected with a metal bell or hood. A later model uses a plunger type transfer mechanism. The plasma is considerably reduced in bulk during transference, and solubility is increased by the granulation. After sterile rubber caps have been applied, a high vacuum is drawn through a needle and the whole top sealed. Deterioration of vacuum is prevented by sealing the rubber cap with celloidin or suitable plastic solution. While we are constructing a dehumidified, air-conditioned, sterile room for our procedures, no difficulty has been encountered with these methods in an ordinary closed small room.

These bottles of plasma, together with enough pyrogen-free water to make the four times concentrate have been kept in our emergency room, in the obstetrical bags, and in the central laboratory. They have also been mailed to distant points in and out of Texas, and they have been used without trouble. This dry form invariably has been preferred to the frozen solution of concentrate which we had formerly used, due to greater availability and speed in preparing for use.

#### SUMMARY AND CONCLUSIONS

1. Mass production methods suitable for national defense needs and proved by extensive routine use are presented.

2. The complete safety of these methods is reflected in a febrile reaction rate of 0.74 per cent.

3. The use of serum separators and other partially open methods essential for volume output is safe under the conditions described.

4. The advantages of desiccated plasma prepared by the adtervac process are discussed.

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## THE ASSAY OF HEPARIN<sup>2</sup>

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A NUMBER of different methods for assaying heparin have been described, some employing whole blood and some plasma. Some of these may be mentioned briefly. Jacques and Charles<sup>7</sup> recently reviewed three methods, namely; (1) a modification of the Howell method by Charles and Scott,<sup>4</sup> in which observations are made on the degree of clotting in samples of cat's blood containing varying amounts of the standard and unknown heparins; (2) a new thrombin assay, also a modification of the Howell method, in which a standardized thrombin is used to clot samples of oxalated beef blood containing varying amounts of the standard and unknown heparins, and (3) the Fiseher and Schmitz method<sup>6</sup> in which the clotting time of chicken plasma on addition of thrombokinase is determined for varying concentrations of unknown and standard heparin. Chargaff<sup>3</sup> devised a method using chicken plasma, similar to that of Fiseher and Schmitz but having the time constant and observing clot formation with varying amounts of heparin, following the addition of thrombokinase (the "musele coagulin" of Fiseher<sup>5</sup>).

In a recent communication, Schütz<sup>9</sup> describes a method in which rabbit's whole blood is added to varying concentrations of standard and unknown heparin solutions. The concentration of heparin in successive tubes is increased by 25 per cent. The comparison is made the following day, and the two racks containing the standard and unknown are moved along until there is a matching of the tubes. In the present method in which beef plasma is used, the concentration of each successive tube is increased by antilog 0.15 (about 40 per cent). With this interval, the clotting range may be covered with only 5 tubes. The standard and unknown tubes are graded according to a procedure to be described later, instead of matching directly as in Schütz and other methods.

The purpose of this paper is not to present a completely new method of assay but rather to discuss a technique of comparing the clots in the standard and unknown tubes employing a method already described by Reinert and Winterstein.<sup>8</sup> Only certain minor modifications have been made in their method in order that a statistical procedure may be applied to the results. Briefly, the method consists in observing the degree of clot formation at 37° C. in tubes containing heparinized citrated beef plasma to which calcium chloride has been added. The anticoagulating unit (a.c.u.) of Reinert and Winterstein is accordingly defined as that amount of heparin which inhibits for four hours the clotting of recalcified citrated beef plasma when kept at a temperature of 37° C. Five of these units equal one Best<sup>1</sup> or Charles and Scott unit, or simply, "Toronto Unit." (Reinert and Winterstein; also the author's evaluation.)

<sup>2</sup>From the Pharmacology Laboratory, Hoffmann-La Roche, Inc.  
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The principles of evaluation described below can of course be applied to methods other than that of Reinert and Winterstein, but their method seems well suited to these principles, and with beef blood more readily available in larger quantities than blood obtained from the cat, rabbit, or chicken, more samples can be run simultaneously.

TABLE I  
AMOUNT OF HEPARIN IN MICROGRAMS TO BE USED IN EACH TUBE  
(Each increment = antilog 0.15)

| TUBE NO. | SERIES 1 | SERIES 2 | SERIES 3 | SERIES 4 |
|----------|----------|----------|----------|----------|
| 1        | 0.71*    | 0.78     | 0.85     | 0.92     |
| 2        | 1.00     | 1.10     | 1.20     | 1.30     |
| 3        | 1.41     | 1.55     | 1.70     | 1.84     |
| 4        | 2.00     | 2.19     | 2.39     | 2.59     |
| 5        | 2.82     | 3.10     | 3.38     | 3.66     |

\*In practice, the nearest twentieth of a microgram is used. This brings the volume measurements to the nearest hundredth of a milliliter.

TABLE II  
VOLUMES OF REAGENTS USED IN SERIES 1  
(Series 2, 3, and 4 are to be arranged similarly. The concentration of heparin is 57/ml.)

| TUBE NO. | PLASMA (ml.) | AMOUNT OF HEPARIN |      | 0.9% SALINE (ml.) | CaCl <sub>2</sub> * (ml.) |
|----------|--------------|-------------------|------|-------------------|---------------------------|
|          |              | γ                 | ML.  |                   |                           |
| 1        | 1            | 0.7               | 0.14 | 0.66              | 0.2                       |
| 2        | 1            | 1.0               | 0.20 | 0.60              | 0.2                       |
| 3        | 1            | 1.4               | 0.28 | 0.52              | 0.2                       |
| 4        | 1            | 2.0               | 0.40 | 0.40              | 0.2                       |
| 5        | 1            | 2.8               | 0.56 | 0.24              | 0.2                       |

\*See text for concentration of calcium.

#### PROCEDURE

Fresh beef blood is collected at a slaughterhouse (or preferably from the living animal by venipuncture) in two 1-liter bottles, each containing 50 ml. of 4 per cent sodium citrate solution. The blood is drawn into each bottle until the 500 ml. mark is reached. The mixed blood thus contains 0.4 per cent sodium citrate. On arriving at the laboratory, the blood is centrifuged for thirty minutes at 2,000 r.p.m. The supernatant plasma is placed in the reservoir of a 10 ml. Koch pipette. One milliliter portions are measured into sulfuric acid-dichromate-cleaned 12 by 100 mm. test tubes. To each tube are added appropriate amounts of heparin solution, 0.9 per cent saline, and calcium chloride solution so that the total volume is always exactly 2 ml. Five milliliter Koch pipettes graduated to 0.02 ml. are used for the latter solutions. The tubes are stoppered with snug-fitting corks paraffined only on the end (otherwise they tend to slip out) and inverted 2 or 3 times. They are then placed in a water bath at 37° C. and examined three hours later.

The standard and unknown heparin solutions are each made up in 0.9 per cent saline to contain 5 gammas of heparin per milliliter. In an assay there are 4 sets of 5 tubes each for each sample. The weights of heparin used in these 20 tubes are shown in Table I. Table II shows the arrangement of the 5 tubes of

the first series. For convenience, charts are used giving the actual burette readings for heparin and saline solutions for each series.

As indicated in Table II, the volume of calcium solution is 0.2 ml. Since different plasmas vary, the amount of calcium required varies and this amount must be determined on each new plasma before the assay is run. To do this, 6 tubes are set up, each containing 1 ml. of plasma, 2 gammas of standard heparin (0.4 ml.) and calcium chloride in the following amounts: 0.8, 1.0, 1.25, 1.5, 2.0, and 2.5 mg. respectively. The total volume in each case is 2.0 ml. The concentration of calcium chloride giving nearest to a 2+ clot (see below) after thirty minutes is selected and used for the assay. Occasionally, a longer time than thirty minutes is necessary. If the time is more than sixty minutes, the test must be repeated, possibly with higher calcium concentrations. From a stock solution of calcium chloride the assay solution is made so that the right amount of calcium is contained in 0.2 ml.

In conducting an assay, the 5 tubes for Series 1 are prepared for the standard heparin and for each unknown sample in succession. Series 2 is then similarly prepared and finally Series 3 and 4. In this way, any changes in the plasma due to standing have a minimum of influence on the results. As many as 6 unknowns can be conveniently run simultaneously with the standard.

#### READING THE TUBES

The reading of the tubes involves the use of a clotting scale based on the proportion of the total possible clot formed in each tube. Complete clotting (usually with marked retraction) is called 4+, and no clot formation is called 0. Intermediate values then are +, 2+, and 3+. On a percentage basis, + = 25 per cent, 2+ = 50 per cent, 3+ = 75 per cent, and 4+ = 100 per cent. The 2+ or 50 per cent clot formation has been adopted as the end point for comparing the standard and unknown heparins. This corresponds to the ED50 (50 per cent effective dose) of the usual dose-effect curve. All values are computed to this basis. It is apparent that not all partial clots will correspond exactly to +, 2+, or 3+, and that many clots will lie at intermediate values. Such intermediate values are designated by changes in the above symbols. For example, a clot which appears to be slightly heavier than 2+ is called 2+(+) or if slightly lighter, 2+(-). A 3+(-) clot would be slightly heavier than a 2+(+). Also, a clot that is not quite complete is called 4+(-), and a very small clot is 0(+). Thus there are 13 classifications, varying (theoretically) by  $8\frac{1}{3}$  per cent. It is not considered practical to attempt a finer classification under present conditions. Jacques and Charles' report: "At least four intermediate stages can be distinguished, and with practice, eight can be detected." This refers to whole blood, whereas, with plasma as in the present method, at least 11 intermediate stages can be ascertained with little difficulty.

One may ask, "Why resort to a clotting scale based on + values rather than one based directly on percentage, since the + values are ultimately converted into percentages?" The 3 principal + values are +, 2+, and 3+, corresponding to  $\frac{1}{4}$ ,  $\frac{1}{2}$ , and  $\frac{3}{4}$  clots. It is easier for the eye to read, for example, "a

half clot plus a little more" [2+(+)] than "58 $\frac{1}{3}$  per cent." In other words, the clot, after first rough appraisal by the eye, is graded more precisely by shifting the + value to one side or the other of the main classification. If a graded series of "standard clots" could be prepared and photographed, the task of reading would be simplified and a percentage scale could be employed. The difficulty arises from the variation of clot characteristics from plasma to plasma. Rate of shrinkage, volume of clot, opacity, flocculation, and even color are the variable factors that meet the eye. For example, a 4+ clot may be shrunken to a small volume or may show no retraction whatsoever at the time of reading.

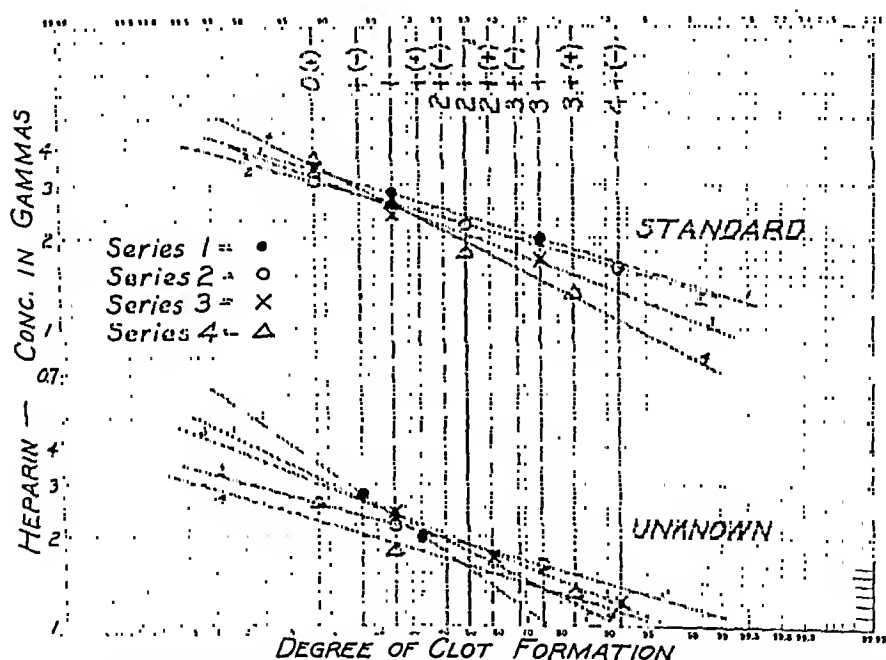


Fig. 1.—Curves showing the relation between the concentration and the degree of clotting on logarithmic probability paper. Solid lines: Best fitting straight lines for data on each set of tubes excepting Series 1 of the unknown. In this case, with only two points, the dashed line is the best fitting line, while the solid line is the more likely since it is more nearly parallel to the general slope.

It is obvious that the unaided inspection of a single tube may not accurately classify it. What might be identified as 2+ at one time may be considered 2+(-) at another. Therefore, the standard series is read first as carefully as possible, and the unknown tubes are then compared to these for grading. The tubes are in no sense paired as in the Schütz method, although frequently a tube from the unknown series may be observed to be identical with one of the standard tubes. Exact pairing facilitates the grading but is not necessarily the true end point.

The clotting process, as here observed, is not an "all or none" response and therefore is subject in a greater degree to the error of personal interpretation. In spite of this, it is believed that the end point here (defined as the formation of 50 per cent of the possible clot) can lead to a more accurate evaluation of anticoagulant activity than heretofore.

## INTERPRETATION OF RESULTS AND CALCULATION OF POTENCY

After values have been assigned to each clot, an estimation of the concentration of heparin corresponding to a 2+ clot may be made by plotting the logarithms of the concentration of heparin against the probits of the graded clots or by plotting directly on logarithmic probability paper. The probits are determined by first converting the + values to percentages and the latter into probits by means of Bliss' table of probits.<sup>2</sup> The use of logarithmic probability paper has been the method of choice as it is more convenient and saves time. The data from a typical experiment are illustrated in Fig. 1. The heavily ruled ordinates correspond to the eleven + values (other than zero and 4+). They were drawn with India ink so as to remain as permanent reference lines while lightly penciled data could be quickly erased. There are usually 3 values that can be plotted (sometimes only 2 and sometimes 4), and the best fitting straight line is then drawn. (If the concentration intervals were less than antilog 0.15, more points would be available for plotting.) The "50 per cent dose" of heparin is indicated by the point at which this line crosses the 2+ ordinate. If only two points are obtained, it may happen that a line drawn through them does not have the usual slope. In such a case (see dashed line, Fig. 1) it should be drawn nearly parallel to the average slopes and between the points rather than through them, giving greater weight to the point nearest the 50 per cent or 2+ line. Slope as well as position must always be considered.

The calculation of potency may be made in a.e.u. (Reinert and Winterstein units) or in "Toronto Units." The following example is worked out in a.e.u. The standard contains 500 a.e.u./mg. (100 Toronto Units). If an estimation on one series shows the standard to have an equivalency of 2.35 gammas for a 2+ clot and the unknown 1.82 gammas, then the potency of the unknown is  $500 \times 2.35 \div 1.82 = 645$  a.e.u./mg. This example, together with the other 3 series, is illustrated in Table III. For a complete assay the entire procedure is repeated on another day with a different sample of plasma. For the heparin assayed in Table III, the other four values were 600, 561, 622, and 614 a.e.u./mg. for the 4 series, respectively. The average for all 8 results was  $595 \pm 12$  a.e.u./mg.

## DISCUSSION

It may be asked if the relationship between the logarithms of the concentration of heparin and the probits corresponding to the percentage clot formation is actually linear or if it is a curved function. This cannot be answered with certainty at the moment, but in any event most of the data are randomly scattered about a straight line. When the majority of the data are not so scattered, the middle points are usually below the best fitting straight line and a curve drawn through them would have its convexity downward. This curving is slight in most cases and is not believed to be significant, but in any event the concurrent use of a standard neutralizes any such errors. Nevertheless, tests are under way to determine more accurately the exact nature of the dose-effect relationship.

A few points concerning the causes of error and variation in heparin assays may be briefly mentioned. There should be a minimum of time elapsing between

securing the blood and centrifuging it. Citrated beef plasma undergoes gradual changes on standing; therefore, a complete assay should be performed in the shortest space of time possible. In Table III it may be noted that the amount of heparin corresponding to a 2+ clot tends to diminish from the first to the fourth series. This apparent trend occurs as frequently in the opposite direction so is probably not significant unless it can be attributed to some changing characteristic of the plasma.

TABLE III.

EXAMPLE OF THE ASSAY OF AN UNKNOWN HEPARIN AGAINST THE STANDARD

| STANDARD HEPARIN          |                 |   | UNKNOWN HEPARIN |   |                                 |
|---------------------------|-----------------|---|-----------------|---|---------------------------------|
| AMOUNT OF HEPARIN (GAMMA) | READING OF TUBE | AMOUNT OF HEPARIN ESTIMATED FOR 2+ CLOT | READING OF TUBE | AMOUNT OF HEPARIN ESTIMATED FOR 2+ CLOT | POTENCY OF UNKNOWN (A.C.U./MG.) |
| 0.7                       | 4+              | 2.35                                    | 4+              | 1.82                                    | 645                             |
| 1.0                       | 4+              |   | 4+              |   |                                 |
| 1.4                       | 4+              |   | 4+              |   |                                 |
| 2.0                       | 3+              |   | +(+)            |   |                                 |
| 2.8                       | +               |   | +(-)            |   |                                 |
| 0.8                       | 4+              | 2.22                                    | 4+              | 1.86                                    | 596                             |
| 1.1                       | 4+              |   | 4+              |   |                                 |
| 1.6                       | 4+(-)           |   | 3+              |   |                                 |
| 2.2                       | 2+              |   | +               |   |                                 |
| 3.1                       | 0 (+)           |   | 0               |   |                                 |
| 0.85                      | 4+              | 2.02                                    | 4+              | 1.88                                    | 537                             |
| 1.2                       | 4+              |   | 4+(-)           |   |                                 |
| 1.7                       | 3+              |   | 2+(+)           |   |                                 |
| 2.4                       | +               |   | +               |   |                                 |
| 3.4                       | 0 (+)           |   | 0               |   |                                 |
| 0.9                       | 4+              | 1.90                                    | 4+              | 1.62                                    | 587                             |
| 1.3                       | 2+(+)           |   | 3+(+)           |   |                                 |
| 1.8                       | 2+              |   | +               |   |                                 |
| 2.6                       | +               |   | 0 (+)           |   |                                 |
| 3.7                       | 0 (+)           |   | 0               |   |                                 |

When the tubes are being filled, especially with the plasma, the rate of delivery must be absolutely uniform and not too rapid. Since plasma is a moderately viscous liquid, a considerable amount adheres to the wall of the burette and drains slowly.

After the tubes are placed into the water bath, they should not be agitated. Any preliminary examination should be avoided or at least made with great care.

The volumes used are relatively small and a slight error in reading, even though no greater than the thickness of a graduation mark, can result in observable variation in the nature of the clot.

Cleanliness of the tubes is essential, the final cleaning being done with strong cleaning solution made of potassium dichromate and concentrated sulfuric acid. After thorough washing with tap and distilled water, the tubes are dried in an oven. If a brush is used in the preliminary cleaning before the acid, scratching of the glass should be avoided. The tubes should have as uniform a bore as possible.

Assays conducted in this laboratory have been on crude extracts as well as on the purified products. The crude extracts have shown potencies averaging about

400 a.c.u. per milligram, whereas the final product has averaged about 525 a.c.u. per milligram. This difference may not be the true difference in potencies since it has been pointed out by Jacques and Charles<sup>7</sup> that salts and other impurities cause variations in the clotting process to such a degree that the true anticoagulant value, due to heparin, may be considerably different than the observed value. Nevertheless, the results on many samples have been uniform within the bounds of reasonable consistency. Assays on 10 consecutive crudes and on finished products are given in Table IV.

TABLE IV

## ASSAYS ON TEN SUCCESSIVE CRUDE AND PURIFIED HEPARIN SAMPLES

(Four tests made on each crude and 8 tests on each purified sample. Each group of 5 tubes comprises a "test.")

| CRUDE HEPARIN   |    | PURIFIED HEPARIN |    |
|-----------------|----|------------------|----|
| A.C.U./MG.      | SM | A.C.U./MG.       | SM |
| 419             | 16 | 595              | 12 |
| 415             | 5  | 509              | 10 |
| 430             | 9  | 521              | 14 |
| 436             | 11 | 522              | 13 |
| 434             | 8  | 518              | 10 |
| 409             | 11 | 513              | 11 |
| 375             | 15 | 512              | 7  |
| 388             | 10 | 536              | 11 |
| 372             | 13 | 530              | 7  |
| 400             | 9  | 518              | 7  |
| Mean 408        |    | Mean 527         |    |
| SD = 23.5 units |    | SD = 25.1 units  |    |

a.c.u., anticoagulating unit; Sm, standard error of the mean; SD, standard deviation.

There is probably no other biologic assay in which greater care must be exercised to obtain consistent results. The utmost in uniformity of manipulative procedure must be maintained at all times. This applies to the cleaning of the tubes and burettes as well as to exactness in measuring volumes, accuracy in preparing solutions, and the maintaining of constant time intervals for each step from start to finish. Surprisingly, however, the time of reading the tubes is of less importance than might be expected. It has frequently been observed that the majority of tubes show no observable change between two hours and four hours, the clotting curve apparently tending to level off after one or two hours. Further growth of clots after two hours, when they did occur, were rarely greater than one degree on the clotting scale. For this reason, the observation time of three hours was adopted as an average, and fifteen minutes one way or the other makes little difference. After twenty-four hours very few additional changes in clotting can be observed unless the tubes have been shaken.

## SUMMARY

A technique has been described for determining the anticoagulant potency of heparin. The method consists in observing the degree of clot formation in recalcified citrated beef plasma to which varying amounts of heparin have been added. The degree of clotting is measured on a suitable clotting scale, after which the data in the form of the logarithms of the concentrations and the probits of the percentage clot formations are plotted to determine the "50 per



cent clotting concentration." The potency of an unknown preparation is estimated by comparing its 50 per cent clotting concentration with that of a standard. An assay is performed in several small series of tests so that a statistical evaluation may be obtained.

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## A SIMPLE CLINICAL METHOD FOR DETERMINING SULFONAMIDES IN BLOOD\*

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IN THE Bratton-Marshall method for determining sulfonamides in blood one has a very accurate and satisfactory method which is simple enough for all familiar with laboratory technique. This is not very time consuming if one has a well-equipped laboratory at hand, but it requires the making up of several quantitative solutions and cannot be carried out at the bedside. In order to make the determination of sulfonamides in the blood possible for clinicians in private practice and for those in small hospitals, a simple kit has been devised in which the reagents are put up in tablet form, with the exception of a small bottle of acetone.

The reagents are essentially the same as those in the Bratton-Marshall method with one major exception. Acetone is used for the precipitation of the blood proteins. After the blood has been shaken with acetone, the coagulated proteins settle out very rapidly, leaving a clear supernatant fluid, an aliquot of which can be used for the test. This avoids filtration and the necessity of carrying funnels and filters and saves considerable time. As the trichloroacetic acid used by Bratton and Marshall is hygroscopic, it has been replaced by oxalic acid in the reagent tablet.

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As the determination of the combined sulfonamides is rarely necessary in practice, the test here described, which determines the free forms only, answers most of our clinical needs.\*

#### APPARATUS

1. Pipette (1) 0.2 c.c. for measuring the blood.
2. Test tubes (2) 100 by 12 mm. marked at 1 c.c. and 3 c.c.
3. Comparator block with 6 color standards.

#### REAGENTS

1. Acetone.
2. Tablet No. 1 containing 30 mg. of oxalic acid.
3. Tablet No. 2 containing 1 mg. of sodium nitrite.
4. Tablet No. 3 containing 5 mg. of ammonium sulfamate.
5. Tablet No. 4 containing 1 mg. of N (1-naphthyl) ethylenediamine dihydrochloride.

In the preparation of the tablets, suerose can be used to increase the bulk. The size of the tablets should not exceed 30 mg. to avoid unnecessary turbidity of the solutions.

TABLE I

| AMOUNT OF PHENOLSULFONPHTHALEIN<br>(C.C.) | AMOUNT OF SULFANILAMIDE INDICATED<br>(MG. PER 100 C.C.) |
|---|---|
| 0.09                                      | 2   |
| 0.18                                      | 4   |
| 0.27                                      | 6   |
| 0.42                                      | 8   |
| 0.60                                      | 10  |
| 0.77                                      | 12  |

#### PROCEDURE

1. Deliver 0.2 c.c. of blood into a test tube.
2. Add acetone to the 3 c.c. mark. Shake and let stand until clear (one to two minutes).
3. Transfer clear liquid into another test tube to the 1 c.c. mark.
4. Add water to the 3 c.c. mark and shake.
5. Add tablets No. 1 and No. 2, shake and let stand for three minutes.
6. Add tablet No. 3, shake and let stand for one minute.
7. Add tablet No. 4, shake, and after one minute compare with color standards.

In the kit mentioned above a color chart is used but where this is unavailable color standards may be made up as follows.

Dissolve 10 mg. of phenolsulfonphthalein in 250 c.c. of N/10 solution of sodium hydroxide. To each of six 100 by 12 mm. test tubes add 5 c.c. of N/10 sodium hydroxide solution and varying amounts of the phenolsulfonphthalein

\*The preparation of reagent tablets by the physician is impractical. At our request, the A. S. Aloe Company, St. Louis, Mo., is supplying these, together with a complete kit for carrying out this test.

solution as indicated in Table I. The test tubes containing the color standards should be sealed and protected from direct sunlight. They should be renewed every six months.

The same procedure and color standards can be used for the determination of sulfanilamide derivatives. The amount of drug indicated by the standards should be multiplied by certain factors, when sulfanilamide derivatives are determined. These factors are 1.37 for sulfapyridine, 1.42 for sulfathiazole, and 1.45 for sulfadiazine.

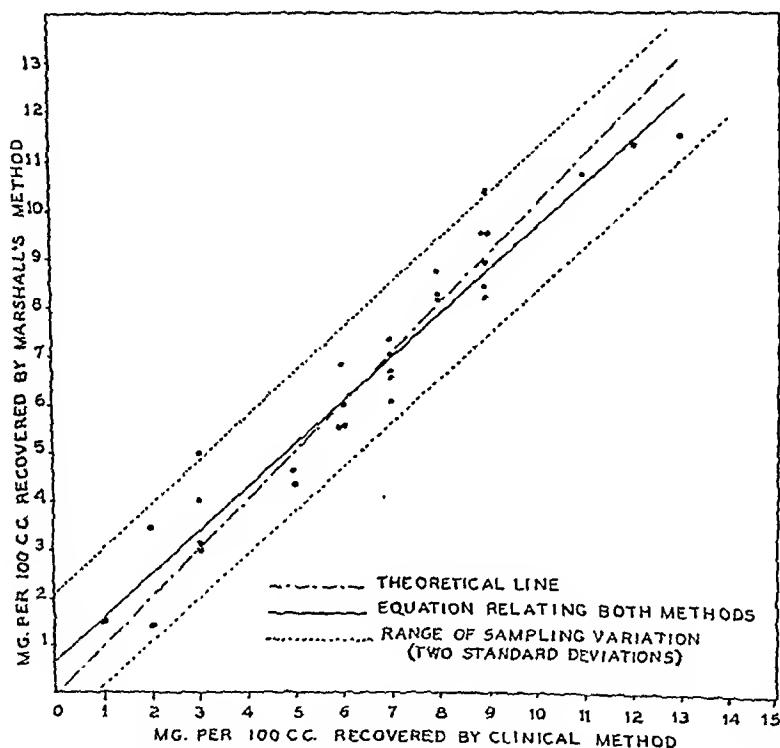


Fig. 1.—Recoveries of sulfanilamide in 30 blood samples. Comparison between the Bratton-Marshall method and the present clinical method.

In order to determine the accuracy of our method, 30 blood samples were analyzed with the Bratton-Marshall method and with our technique. As indicated in Fig. 1, our recoveries follow closely the ones obtained by the Bratton-Marshall method.

#### SUMMARY

A simple clinical method for determining free sulfanilamide and derivatives in the blood has been presented. All of the equipment required for the procedure can be placed in a small kit, and the test can be performed without laboratory facilities.

#### REFERENCE

1. Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination, *J. Biol. Chem.* 128: 537, 1939.

## PROTHROMBIN STUDIES USING RUSSELL VIPER VENOM\*

### III. EFFECT OF LECITHINIZED VENOM ON PROTHROMBIN CLOTTING TIME

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THE use of Russell viper venom (*Daboia* venom) as a thromboplastin-like substance has been reported by many investigators.<sup>1, 2</sup> More recently this venom has been employed, instead of brain extract, as the thrombokinase in the determination of the prothrombin clotting time.<sup>3, 4</sup>

The coagulant action of Russell viper venom is potentiated by the addition of lecithin. Trevan and Macfarlane<sup>5</sup> found that the hemostatic action of Russell viper venom was accelerated by the addition of small quantities of tissue extract or lecithin.

Leathes and Mellanby<sup>6</sup> reported that lecithin from brain or egg yolk had the property of increasing the activity of the thrombokinase in *Daboia* venom.

Witts and Hobson<sup>7</sup> compared the prothrombin clotting times of 43 human beings using Russell viper venom (dilution 1:50,000) and lecithinized venom (adding 0.05 c.c. of a 10 per cent alcoholic solution of ovolecithin to each cubic centimeter of venom solution). The clotting times with lecithinized venom were shorter and less scattered than with venom alone. The usual time was eleven seconds with a suggested range of nine to thirteen seconds. With venom alone the clotting times were from two to nineteen seconds longer. In prothrombin deficiency, coagulation remains impaired despite the addition of lecithin to venom. They concluded that, if venom is used in the prothrombin clotting time test, it must be fortified by the addition of lecithin.

In a later paper Hobson and Witts<sup>8</sup> stated that Russell viper venom acts as a thrombokinase and accelerates the coagulation of blood or plasma in the presence of calcium and that the optimal concentration of the venom for use in the prothrombin clotting time test is 1:20,000. They conclude that, if Russell viper venom is used alone, the coagulation time may be affected by hemolysis, lipemia, the number of platelets *in vivo*, and, in certain circumstances, the speed and duration of centrifugalization, and that these inconsistencies are eliminated by the addition of lecithin to the venom.

Crosbie and Scarborough<sup>9</sup> reported on coagulation studies with Russell viper venom alone and with venom plus a 10 per cent alcoholic solution of ovolecithin (B.P.C.†). Venom, in the absence of hemolysis and without lecithin,

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†The British Pharmaceutical Codex, 1924, p. 757.

is a more potent source of thromboplastin than is brain extract. When lecithinized venom was used, the coagulation times were shorter and the scatter was reduced.

Edsall<sup>10</sup> has reported that the coagulant action of Daboia venom appears to be exerted through an interaction with tissue extracts or with the cephalin contained in such extracts. Only a slight acceleration of coagulation was observed on employing 4 mg. of lecithin, comparable to that obtained with 0.002 mg. of cephalin. This may properly be ascribed to the unavoidable residual contamination of the lecithin with cephalin.

#### METHOD OF STUDY

Blood of patients in the hospital and of patients attending an outpatient clinic was obtained. Blood from patients, both with and without liver disease, was used. Four and one-half cubic centimeters of venous blood were drawn into a dry syringe and mixed with 10 mg. of potassium oxalate in a centrifuge tube. The blood was centrifuged at 1,500 r.p.m. for five minutes, and the oxalated plasma was drawn off. The test was performed within two hours after withdrawal of the blood, and, if hemolysis was present, the sample was discarded. The test was performed as follows: Two-tenths cubic centimeter of oxalated plasma was pipetted into a small test tube (75 by 10 mm.) and 0.2 c.c. of Russell viper venom,\* 1:10,000 solution, was added. Calcium chloride solution (1.11 Gm. calcium chloride per 100 c.c.), 0.2 c.c., was then added, and the stop watch was started. The tube was agitated for ten to fifteen seconds in a water bath (37° C.), then removed and tilted until separate discrete fibrin particles could be seen. The first appearance of the fibrin particles was taken as the end point.

Prothrombin clotting time was determined on the undiluted plasma (100 per cent) and on 40 per cent plasma, diluted with physiologic saline solution.

The test was repeated, using the same plasma but with lecithinized venom. A 10 per cent alcoholic solution of lecithin† (egg) was used in the amount of 0.05 c.c. per cubic centimeter of venom. Venom of the same batch was used in both of the series of tests using venom and lecithinized venom. Each test was performed in triplicate, and the average time was taken for the result. The prothrombin clotting time in normal persons, by this method, for the 100 per cent plasma, is 20.76 seconds while that for the 40 per cent plasma is 33.10 seconds.<sup>11</sup>

#### RESULTS OF STUDY

The routine use of venom and lecithinized venom was carried out on 30 patients, and the resulting data on the 100 per cent plasma are outlined in Fig. 1 with the diagnosis of each patient. The complete data of the 100 per cent and 40 per cent plasma using venom and lecithinized venom are plotted in Fig. 2.

In the undiluted plasma studies the spread obtained with venom alone was from fifteen to thirty-four seconds, while with lecithinized venom it was from

\*Supplied as 'Stypven' Russell viper venom by Burroughs Wellcome & Co., Inc., New York, N. Y.

†Lecithin (Merck).

thirteen to thirty seconds. In the 40 per cent plasma studies the spread obtained with venom alone was from twenty-seven to forty-seven seconds, while with lecithinized venom it was from 17.5 to 34 seconds.

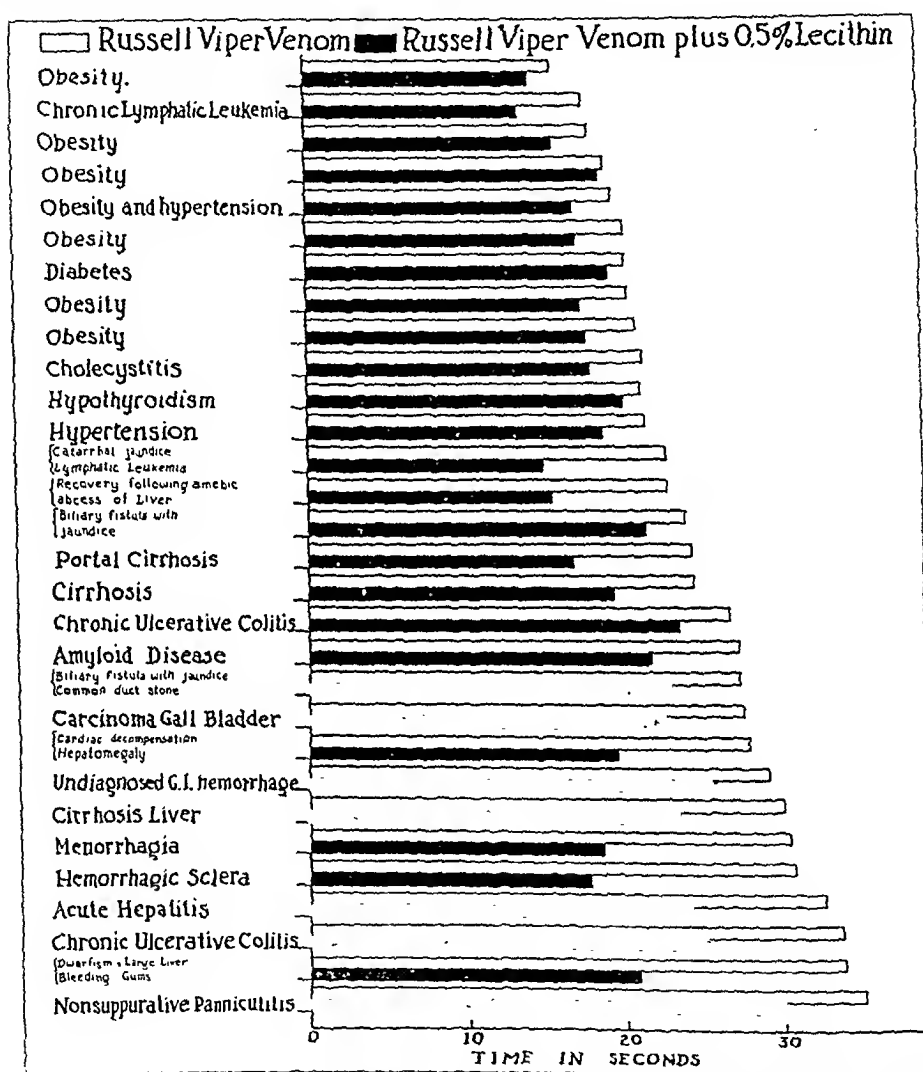


Fig. 1.—Bar graph showing comparison between prothrombin clotting times obtained with Russell viper venom alone and with Russell viper venom plus 0.5 per cent lecithin.

#### DISCUSSION

The addition of lecithin to Russell viper venom decreased the prothrombin clotting time in every instance (Figs. 1 and 2). An estimation of the extent by which lecithin shortened the clotting time was obtained as follows. The clotting times in which venom alone served as the thromboplastic substance were plotted against the corresponding times obtained with lecithinized venom and the equation for a straight line was calculated by the method of least squares. For undiluted plasma this was found to be  $y = 5.74 + 0.554x$  where  $y$  represents the

expected clotting time for lecithinized venom and  $x$  represents the clotting time for venom alone.

Using this equation, one may obtain an idea of how effectively lecithin may be expected to reduce the clotting time. Substituting a value of twenty seconds for  $x$  one finds that the corresponding  $y$  value is 16.8 seconds, or, in other words, the presence of lecithin has reduced the expected clotting time by 16 per cent. If a greater clotting time, thirty-five seconds, is selected for  $x$ , the corresponding  $y$  value is 25.1 seconds and the reduction is 29 per cent. The percentage of reduction, therefore, is not constant but appears to be greater with prolonged clotting times. In the above discussion it must be borne in mind that the calculated values are the expected average values and the assumption was made that the data could be fitted by a straight line.

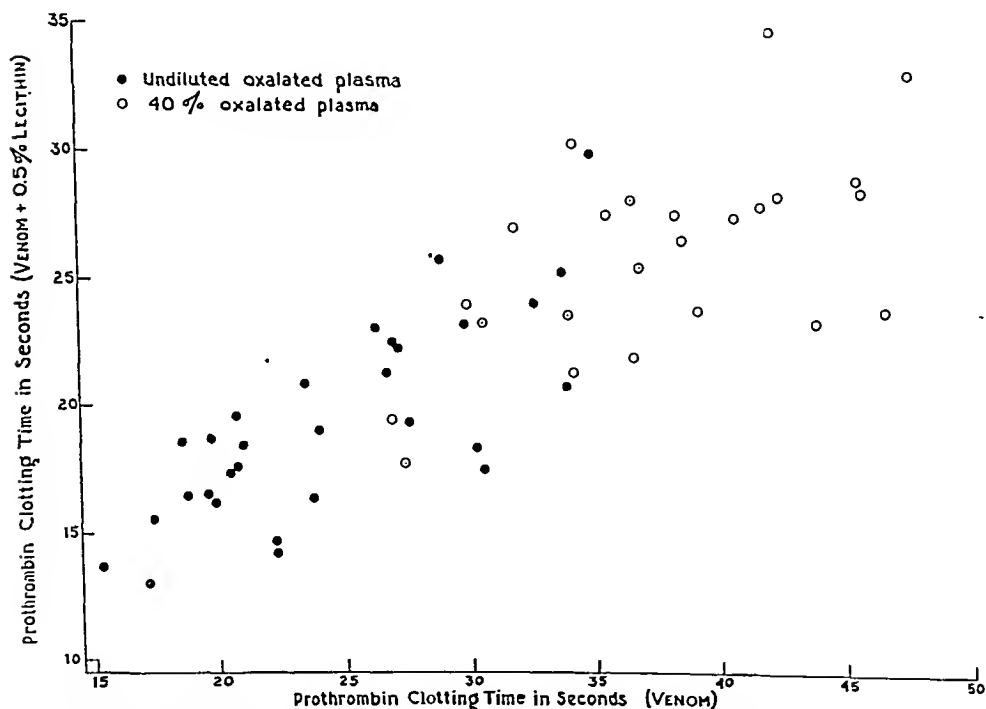


Fig. 2.—Comparison between prothrombin clotting time tests done with Russell viper venom alone and with venom plus 0.5 per cent lecithin on undiluted oxalated plasma and on 40 per cent oxalated plasma diluted with physiologic saline.

Certain prothrombin clotting times (Fig. 1) which would be classed as abnormal with venom alone and presumably by Quick's test<sup>4</sup> would fall into the normal range when lecithinized venom is used. For example, one patient with dwarfism, large liver, and bleeding gums (Fig. 1) had a prothrombin clotting time of 33.7 seconds with venom alone and 20.7 seconds with lecithinized venom. Another patient with hemorrhagic sclera (Fig. 1) had a prothrombin clotting time of 30.4 seconds with venom alone and 17.5 seconds with lecithinized venom. A third patient with menorrhagia (Fig. 1) had a prothrombin clotting time of 30.1 seconds with venom alone and 18.3 seconds with lecithinized venom. In each of these three patients the prothrombin clotting time with venom alone was

prolonged, indicating prothrombin deficiency, while with lecithinized venom the prothrombin clotting time was in the range of presumably normal prothrombin times (with venom alone), i.e., 20.76 seconds  $\pm$  2.3 seconds.<sup>11</sup>

Lecithinized venom may fail to reveal moderate grades of prothrombin deficiency and, therefore, it is desirable to perform the prothrombin clotting time determination with venom alone. However, the use of lecithinized venom may prove to be a valuable adjunct, especially when more information has been secured as to the reasons for the difference between the prothrombin clotting times obtained with venom alone and with lecithinized venom.

#### SUMMARY

Prothrombin clotting time studies using a modified Quick's method employing Russell viper venom (Daboia venom) instead of brain extract have been carried out on 30 human beings.

Undiluted plasma (100 per cent) and 40 per cent plasma were used with venom alone and with venom which had been lecithinized by adding 0.05 c.c. of a 10 per cent alcoholic solution of lecithin (egg) to each cubic centimeter of venom.

The addition of lecithin to the venom accelerated the prothrombin clotting time in each plasma studied.

The percentage of reduction in the prothrombin clotting time is greater in plasma with prolonged clotting times than in plasma with rapid clotting times.

The prothrombin clotting time determination using Russell viper venom should be performed with the venom alone, and it is suggested that lecithinized venom be used as a supplementary procedure. The use of venom alone may reveal certain prothrombin deficiencies which are not evident when the venom is lecithinized. The use of lecithinized venom in some cases may prove to be a valuable adjunct, especially when more information has been secured as to the reasons for the difference between the prothrombin clotting times obtained with venom alone and with lecithinized venom.

We wish to express our appreciation for the cooperation of Dr. Louis Bauman in whose laboratory this work was done.

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# DETERMINATION OF TRYPSIN IN DUODENAL CONTENTS WITH THE EVELYN COLORIMETER\*

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WITH THE TECHNICAL ASSISTANCE OF HERMAN SIPLET, A.B.

DIFFERENT methods for the determination of trypsin in duodenal contents have been proposed. Some of them are based on the estimation of the nonprotein nitrogen in the protein-free filtrate after the digestion of casein with the duodenal contents (McClure<sup>1</sup>). Others determine the amount of amino acids in the filtrate by the Soerensen titration method (Leubner<sup>2</sup>). Hollander<sup>3</sup> and Lueders<sup>4</sup> measure the time in which a certain quantity of amino acids is formed in a gelatin solution. According to the law of Hedin, this time is inversely proportional to the concentration of the enzyme.

Anson and Mirsky<sup>5</sup> have developed a method for proteolytic enzyme determination in which they use a hemoglobin solution as a protein substrate and after digestion determine the amount of those substances which give a blue color with the phenol reagent of Folin and Ciocalteu.<sup>6</sup> The quantity of these substances formed during the incubation is a function of the enzyme concentration. Beazell, Schmidt, Ivy, and Monaghan<sup>7</sup> have adapted the pepsin method of Anson and Mirsky for the determination of pepsin in gastric juice. The trypsin method of Anson and Mirsky is likewise applicable for clinical purposes, and we shall describe the method as we have modified it for use with the Evelyn colorimeter.

The duodenal contents are centrifuged and diluted with physiologic saline solution 1:50. The assay of the enzyme should be performed as soon as possible after collection of the test material. In any case it should not be kept overnight. It has been our experience that during twenty-four hours about 30 per cent of the tryptic activity is lost even if the drainage material is kept in the refrigerator.

## PREPARATION OF THE HEMOGLOBIN SUBSTRATE

The hemoglobin solution contains 2 per cent denatured hemoglobin and is buffered at a pH of 7.5. The solution is prepared according to the method of Anson.<sup>8</sup> Commercial products, such as Baetohemoglobin, give blank values which are too high, and therefore they should not be used.

## INCUBATION

Five cubic centimeters of the hemoglobin solution are pipetted into test tubes. The tubes are stoppered with rubber stoppers and placed in a water bath, heated to 37.5° C., for about fifteen minutes to bring the hemoglobin solution to the right temperature. Then 1 c.c. of the diluted duodenal drainage is

\*From the Medical Research Laboratory Samuel S. Fels Fund, Philadelphia.  
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added in duplicate tubes. The contents of the tubes are mixed thoroughly by inverting several times. Then the tubes are replaced in the water bath. After fifteen minutes they are removed, and 10 c.c. of a 5 per cent solution of trichloroacetic acid are added from another test tube into which it had been measured previously. The solutions are well mixed by pouring the contents back and forth from one tube to the other several times. The stoppers are replaced and the tubes turned upside down repeatedly.\* To get a clear filtrate, the tubes must stand for about fifteen minutes. The solutions are then filtered through a Whatman filter No. 3. Three cubic centimeters of the filtrate are transferred into a colorimeter tube, and 20 c.c. of distilled water are added.

#### PREPARATION OF THE BLANKS

Blanks are prepared to determine the amount of preformed color-giving substances in the duodenal contents and the hemoglobin solution. To 5 c.c. of the hemoglobin solution 10 c.c. of 5 per cent trichloroacetic acid are added. The tubes are stoppered and well shaken. Then 1 c.c. of the diluted duodenal juice is added, and the tube contents are thoroughly mixed. After fifteen minutes the solution is filtered. The filtrate of the blanks is treated the same way as the filtrate of the incubated samples.

#### PREPARATION OF THE STANDARDS

A stock solution of tyrosine is made by dissolving 15 mg. of tyrosine Pfanstiehl (which has been dried in vacuo over sulfuric acid) in 100 c.c. of water containing 0.5 per cent formalin. Before use the stock solution is diluted with distilled water 1:10. To a colorimeter tube are added 15 c.c. of distilled water, 5 c.c. of the diluted tyrosine solution, and 3 c.c. of 0.2 normal hydrochloric acid. The tyrosine stock solution can be kept for about two weeks in the refrigerator without change in strength.

#### DEVELOPMENT OF THE COLOR

To every colorimeter tube, including incubated samples, blanks, and standards, are added 1 c.c. of 3.85 normal sodium hydroxide and 1 c.c. of the phenol reagent (according to Folin and Ciocalteu).† The color reaches its maximum intensity in ten minutes and remains unchanged for a ten-minute period, during which the colorimeter readings should be made.

For adjusting the galvanometer scale to 100 the following "reading blank" is used. Into a colorimeter tube are pipetted 20 c.c. of water, 3 c.c. of 0.2 normal hydrochloric acid, 1 c.c. of 3.85 normal sodium hydroxide, and 1 c.c. of the phenol reagent.†

After the galvanometer scale has been adjusted to 100, the reading blank is replaced by the standard tube, and its color value is determined. It is advisable to read standards with every group of unknown samples as the phenol reagent is not absolutely stable and slight changes in it may occur. The Rubicon filter No. 660 is used. The calibration curve shows that this filter gives results in accordance with the logarithmic law of Lambert and Beer. After the color value of the standard has been determined, all incubated samples and blanks are read.

\*In order to precipitate any of the material adhering to the stoppers.

†The tubes are stoppered and the contents are mixed by inversion.

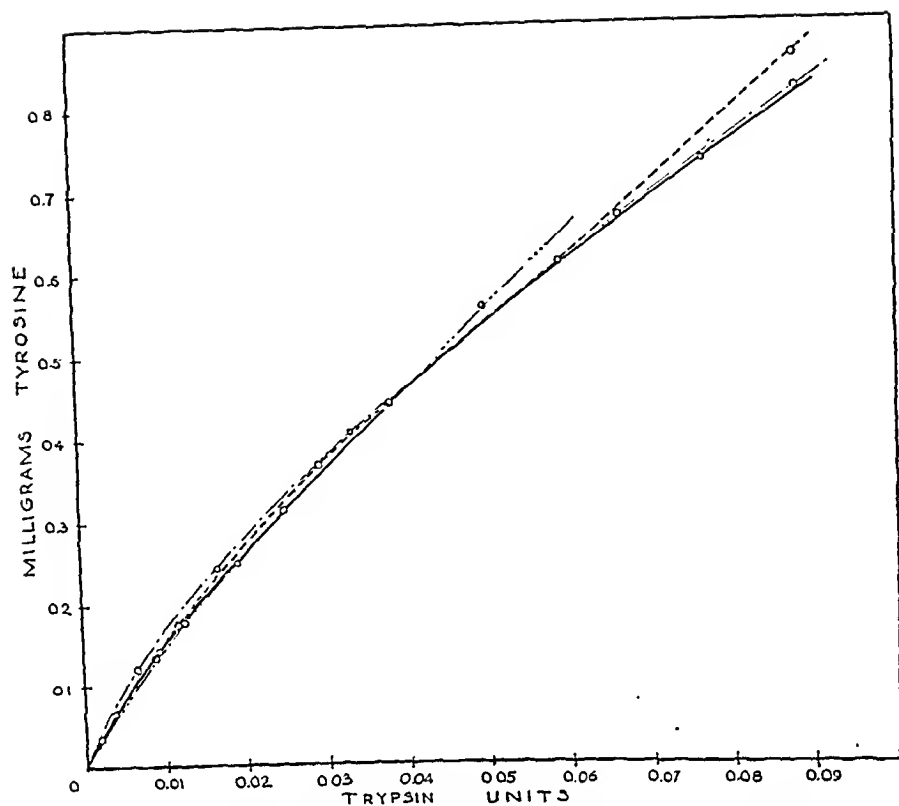


Fig. 1.—Four activity curves made from different duodenal contents.

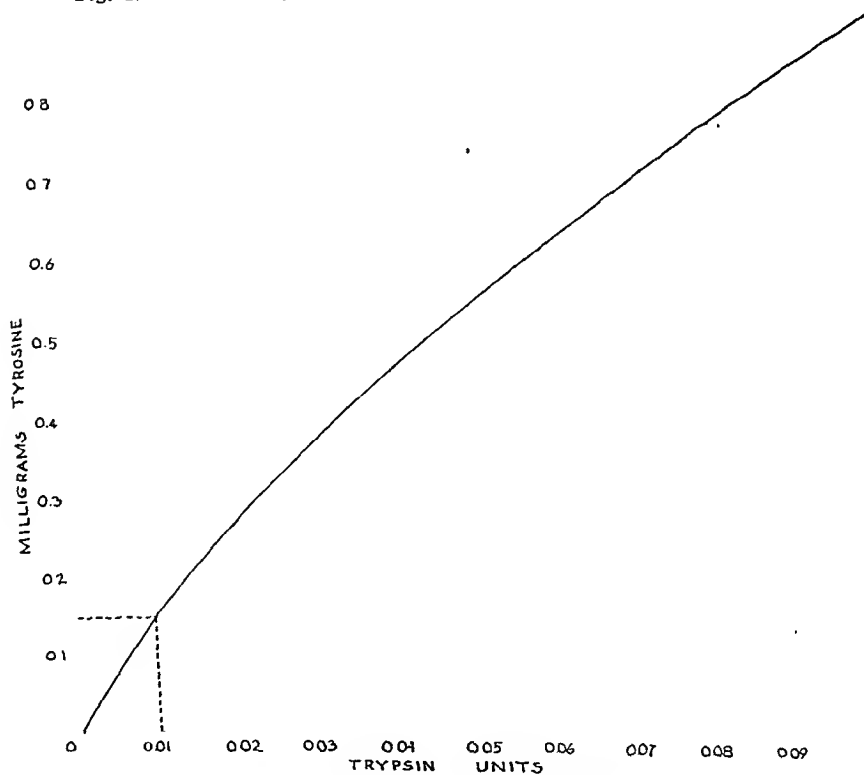


Fig. 2.—Standard activity curve represents the average of the four curves of Fig. 1.

## CALCULATIONS OF THE RESULTS\*

$K = \frac{L}{0.075}$ .  $K$  is the calibration constant;  $L$  is the photometric density of the standard; 0.075 is the tyrosine content of the standard in milligrams.

The quantity of tyrosine developed by 1 e.e. of the diluted sample is formed from the equation  $C = \frac{L_1 - L_2}{K} \times \frac{16}{3}$ .  $L_1$  is the average photometric density of the duplicate incubated samples.  $L_2$  is the photometric density of the blank.  $\frac{16}{3}$  refers to the dilution.  $C$  = milligrams of tyrosine per cubic centimeter of the diluted duodenal material.

## TRYPSIN UNITS

For our definition of a trypsin unit we have applied the definition which Beazell, Schmidt, Ivy, and Monaghan have given for a pepsin unit. Accordingly, one trypsin unit represents such tryptic activity which develops an equivalent of 1.0 mg. of tyrosine per minute under the conditions described. The results are expressed in trypsin units per 100 e.e. of duodenal material. For the conversion of the milligrams of tyrosine into trypsin units an activity curve was constructed in the following manner. Various dilutions of highly active duodenal contents were incubated with the hemoglobin solution; the developed quantities of tyrosine were plotted as ordinates, and the corresponding concentrations of the duodenal contents, as abscissae. Four such "activity curves" were then constructed from four different duodenal contents; in every curve the point in the abscissa corresponding to 0.15 mg. of tyrosine on the ordinate was marked to find the dilution of the duodenal content which corresponds to  $\frac{1}{100}$  of a trypsin unit. From this the abscissa values were recalculated and expressed in trypsin units. The curves were thus reconstructed and brought into one coordinate system (Fig. 1). Fig. 2 represents the average curve of the four curves and can be used as a standard curve for converting the tyrosine values into trypsin units. Multiplication by 5,000 gives the trypsin units per 100 e.e. of the undiluted duodenal material.

The estimations performed in duplicates on 57 specimens show an average difference of 2.2 per cent between the duplicates.

## CONCLUSION

A method has been described for the determination of tryptic activity in duodenal material by means of the Evelyn photocolormeter.

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\*For details see "Notes on Operation of the Evelyn Photoelectric Colorimeter" issued by the Rubicon Company, Philadelphia.

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## AN ADAPTER FOR MICROCENTRIFUGE SHIELDS\*

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IN MICROANALYTICAL procedures the use of very small centrifuge tubes is not infrequently required. These tubes from 0.5 ml. to 3 ml. capacity must be suspended in correspondingly small holders called shields. We believe that the most commonly used type is the Cornell style,  $\frac{1}{2}$  inch outside diameter and about  $2\frac{1}{4}$  inches long.† Even the smaller type centrifuges equipped with standard heads will not support these small shields but require a special head, costing several dollars. The larger floor type centrifuges which are most commonly used in the laboratory require an additional adapter to attach the microhead to the shaft.

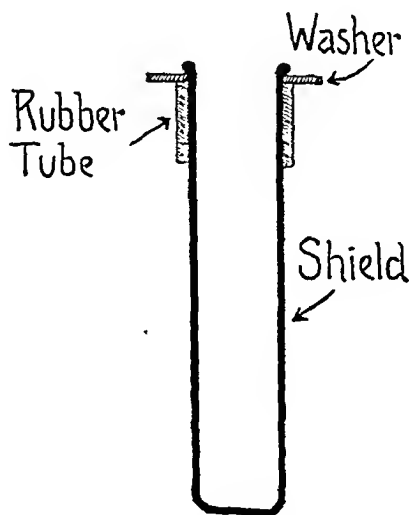


Fig. 1.

A very simple adapter is described which permits the use of the microshields with the ordinary head and shields for the 15 ml. centrifuge tubes.

\*From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee.

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†E. H. Sargent & Company, catalogue No. S17195.

Fig. 1 illustrates the arrangement. An ordinary iron washer of  $\frac{7}{8}$  inch outside diameter is drilled out until the microshield can just be pushed through, a so-called sliding fit. After the washer has been pushed up until it touches the ring of the shield, it is held in place by a piece of heavy walled rubber tubing of  $\frac{5}{16}$  inch inside diameter and  $\frac{3}{32}$  inch wall. The pieces of tubing should be of exactly the same length for all of the shields used in order to equalize their weight. The rubber tube is slipped over a round piece of material, such as a dowel, is marked in  $\frac{1}{2}$  inch lengths, and is cut with a rolling motion with a very sharp knife. The washers must, of course, be of the same thickness and should be balanced before they are attached to the shields. Our shields with the adapter have the same weight within a range of 0.2 Gm.

#### SUMMARY

An easily made adapter for microcentrifuge shields is described. The changing of centrifuge heads is obviated.

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### THE DIRECT BIURET METHOD FOR THE DETERMINATION OF SERUM PROTEINS AS APPLIED TO PHOTOELECTRIC AND VISUAL COLORIMETRY\*

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FURTHER investigation of the direct biuret method for the determination of serum proteins has led to several important refinements of the original procedure.<sup>1</sup> As modified, the method is more accurate, requires less time and fewer precautions than formerly, and is adaptable to visual as well as photoelectric colorimetry. An increase in concentration of the components of the biuret reagent permits accurate determination of serum protein up to 11 per cent. The use of a ready-mixed biuret reagent prevents precipitation of protein or eupric hydroxide. Such precipitation may occur if strong sodium hydroxide and copper sulfate solutions are improperly mixed in the presence of protein. The formation of turbidity, at times a complicating factor in the original method, is delayed by the addition of ether to the reaction mixture. Development of the biuret color under these controlled conditions is rapid, and readings may be made immediately after ether extraction and centrifugation. The improved direct method requires only ten minutes for the determination of serum total protein and twenty to twenty-five minutes for a protein fractionation.

#### REAGENTS

*Biuret Reagent (TP) for Total Protein.*—To 500 c.c. of 14 per cent sodium hydroxide ("carbonate free") in a pyrex bottle or flask, add 100 c.c. of 1 per cent copper sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) solution and mix. Protect from dust and other contaminants. A rubber stopper may be used.

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\*From the Division of Biochemistry, Laboratories, Philadelphia General Hospital, Philadelphia).

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*Biuret Reagent (ALB) for Albumin.*—To 300 c.c. of 23 per cent sodium hydroxide ("carbonate free") in a pyrex bottle or flask, add 100 c.c. of 1 per cent copper sulfate solution and mix.

*Ether.*—U.S.P. grade is satisfactory.

*Sodium Chloride.*—0.9 per cent.

*Sodium Sulfate.*—23 per cent.

*Standard Serum (for Calibration).*—Obtain 5 to 10 c.c. of a single or mixed specimen of normal serum free from cells and hemolysis. Determine the protein concentration in duplicate samples by the macro- or micro-Kjeldahl method. Let C represent grams per cent total protein.

#### METHOD FOR PHOTOELECTRIC COLORIMETRY

*Standardization.*—Prepare stock standards in duplicate by measuring 1 c.c. portions of the analyzed serum into 10 c.c. volumetric flasks and diluting to the mark with 0.9 per cent sodium chloride. Mix. Prepare in duplicate a series of dilute protein standards by adding varying amounts of the stock standard and 0.9 per cent sodium chloride, as indicated in Table I, to 4 c.c. of the biuret

TABLE I

| STOCK STANDARD<br>(c.c.) | 0.9 NaCl<br>(c.c.) | PROTEIN EQUIVALENT (C)<br>(GM./100 c.c.) |
|--------------------------|--------------------|--|
| 0.1                      | 2.0                | 0.1C                                     |
| 0.2                      | 1.9                | 0.2C                                     |
| 0.5                      | 1.6                | 0.5C                                     |
| 0.7                      | 1.4                | 0.7C                                     |
| 1.0                      | 1.1                | 1.0C                                     |
| 1.2                      | 0.9                | 1.2C                                     |
| 1.5                      | 0.6                | 1.5C                                     |
| Blank                    | 2.1                | 0.0                                      |

reagent (ALB) as prepared for albumin determinations. Add 2 c.c. of ether to each tube, stopper, shake vigorously for approximately ten seconds, centrifuge for five minutes at about 2,500 r.p.m., and read in a photoelectric colorimeter. The readings of standard solutions are plotted against concentration of protein represented by each standard. From the graph so obtained colorimetric readings can be converted into concentration of protein. As an alternative, the concentration of protein in an unknown serum can be calculated by substituting the readings of the unknown and standard sera as obtained in the biuret total protein determination and the protein concentration of the standard in the equation  $X = (U - B) \frac{C}{R - B}$ . X equals the grams of protein in 100 c.c. of the unknown serum; U, the reading of the unknown; B, the reading of the blank; C, the grams of protein in 100 c.c. of the standard serum; and R, the reading of the standard serum.  $\frac{C}{R - B}$  need be determined only once (in duplicate) for each standard serum and will remain constant unless mechanical or other changes occur in the photoelectric colorimeter. The calibration should be verified periodically with fresh standard serum.

*Total Protein.*—To exactly 6 c.c. of biuret reagent (TP) in a photoelectric colorimeter tube add 0.1 c.c. of fresh serum (free from cells and hemolysis)

with a micropipette after wiping the outside of the pipette. Rinse the pipette three or more times with the biuret reagent. Add 2 c.c. of ether, stopper, shake vigorously for approximately ten seconds, remove the stopper, and immediately centrifuge at about 2,500 r.p.m. for five minutes. Read in a photoelectric colorimeter within ten minutes after centrifuging. A green filter transmitting light between 500 and 570  $m\mu$  with maximum transmission at 540  $m\mu$  is satisfactory. The reading of the blank (Table I) is constant at 65-69 on the Klett-Summerson photoelectric colorimeter and needs to be determined only once for each new set of reagents.

If the serum is clear and contains only traces of hemoglobin and no more than normal concentrations of other pigments, fairly accurate results are obtained without ether extraction and centrifugation if readings are made five minutes after the development of the biuret color. When ether is not used, carefully mix the solutions by inverting; avoid shaking.

*Albumin.*<sup>3</sup>—Add 0.5 c.c. of serum to 7.5 c.c. of 23 per cent sodium sulfate in a test tube (18 by 120 mm.) and mix thoroughly by inverting. Add about 3 c.c. of ether and shake vigorously for twenty to thirty seconds. Cap or stopper the tube and centrifuge five to ten minutes at about 2,200 r.p.m. After centrifugation, slant the tube so that the tightly packed globulin precipitate separates from the walls of the tube. Insert a pipette through the ether layer preferably along the lower wall, withdraw 2 c.c. of the albumin solution, add to 4 c.c. of the biuret reagent (ALB) in a photoelectric colorimeter tube, and mix immediately. Then add 2 c.c. of ether and proceed as in the biuret method for total protein. Under certain conditions the use of ether may be omitted as stated above. To calculate the amount of albumin present, determine the protein equivalent of the reading by means of the graph or equation obtained by the standardization and multiply by 0.784.

*Globulin.*—Total protein minus albumin equals globulin.

#### METHOD FOR VISUAL COLORIMETRY

*Standard I.*—Add 1 c.c. of standard serum of known protein content and 1 c.c. of 0.9 per cent sodium chloride solution to 140 c.c. of biuret reagent (TP) and mix.

*Standard II.*—Add 2 c.c. of standard serum to 140 c.c. of biuret reagent (TP) and proceed as for Standard I.

*Standard III.*—Add 3 c.c. of standard serum to 139 c.c. of biuret reagent (TP). These standards will keep at least one month if stored in a pyrex bottle in a refrigerator.

For the determination of protein proceed as in the photoelectric method. Shake 6 c.c. of the standard biuret solutions with 2 c.c. of ether and centrifuge simultaneously with the unknowns. Remove sufficient colored solution from beneath the ether layer by means of a pipette and compare in a colorimeter against both a higher and a lower standard at room temperature. Use a green filter (maximum transmission at 540  $m\mu$ ) in the colorimeter eyepiece. If the reading is within 10 to 15 per cent of the reading of one standard, the comparison with the other standard may be omitted.



*Calculation.*— $X = \frac{A \frac{r_a}{10} + B \frac{r_b}{10}}{2}$  where X is the protein concentration of

the unknown in grams per 100 c.c.; A and B, the protein concentrations of lower and higher standards in grams per 100 c.c. of the standard serum; and  $r_a$  and  $r_b$ , the readings of lower and higher standards when the unknown is set on the left at 10 mm. A similar use of two standards has been described by Goudsmit<sup>4</sup> for the determination of creatinine by means of a photoelectric colorimeter.

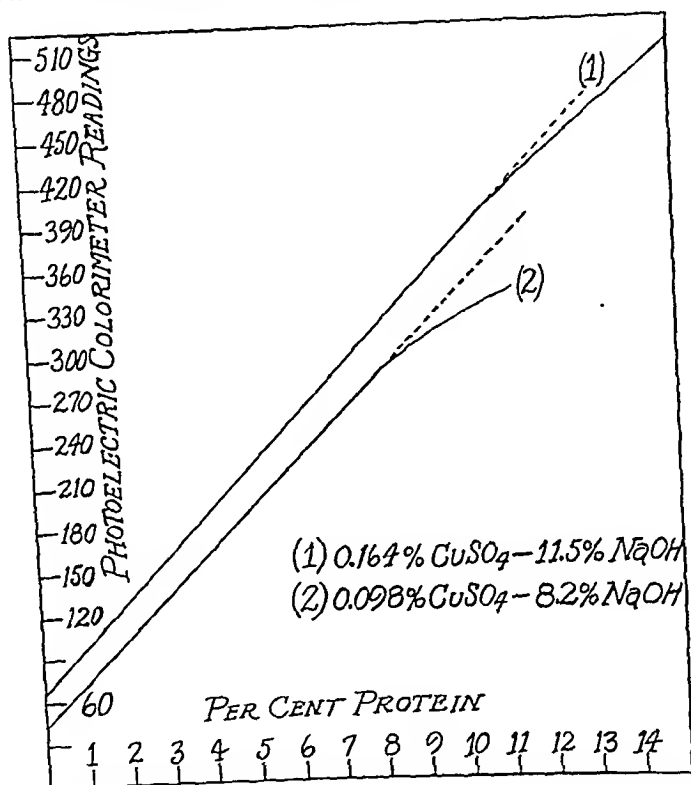


Fig. 1.—Biuret color intensity at increasing protein levels and the effect of different concentrations of copper sulfate and sodium hydroxide.

#### DISCUSSION

The biuret reaction mixture employed contains approximately 11.5 per cent sodium hydroxide and 0.16 per cent copper sulfate, concentrations that are considerably higher than those originally used.<sup>1</sup> When copper sulfate is increased to this strength, the ratio of protein concentration to biuret color produced is linear up to 11 per cent protein as shown in curve 1 (Fig. 1). Curve 2 represents the biuret color produced with the approximate concentration of reagents originally used. Increase in sodium hydroxide concentration also is necessary to avoid the precipitation of cupric hydroxide, especially on standing. Concentrations of sodium hydroxide as high as 13 per cent can be used, but 11.5 per cent was found to be sufficient for a stable reaction mixture. The presence of carbonate in the biuret reagent may cause precipitation with a

decrease in color. For this reason sodium hydroxide solutions were prepared from clear 70 to 75 per cent solutions which had stood until sodium carbonate had separated.

Robinson and Hogden<sup>2</sup> stated that in the direct biuret method<sup>1</sup> the solutions were not perfectly clear, a straight line relationship did not continue above 8 per cent protein, and results comparable to their indirect method were obtained only by the most rigorous control of the time of reading; these objections do not apply to the modified procedure. Several of the time-consuming operations of the indirect method of Robinson and Hogden are avoided in the direct method since their procedure requires precipitation of protein with trichloroacetic acid, the redissolving of protein precipitate with sodium hydroxide, and the removal of excess cupric hydroxide by centrifugation.

When ether is used, careful control of time of reading is unnecessary as solutions remain clear for about twenty minutes after centrifugation. On longer standing turbidity does develop, especially in some cholemic sera. However, if the solutions are again shaken with ether and centrifuged, clear solutions are obtained with unchanged protein values even though the solutions have stood for several hours.

The increased amount of copper employed results in a higher blank, and, at lower concentrations of protein, proportionately greater amounts of reagent uncombined with protein are present. The greater part of the light absorption of the excess biuret reagent is removed by the 540  $m\mu$  filter. Probably for this reason the 540  $m\mu$  filter has proved to be superior (in my experience) to the 560  $m\mu$  filter recommended by Robinson and Hogden.<sup>2</sup> However, if a visual colorimeter is used, it is necessary to employ a series of standards representing approximately 3, 6, and 9 per cent protein. The greater ease of operation and accuracy of the photoelectric colorimetric method makes its use preferable.

The 540  $m\mu$  Klett-Summerson filter absorbs all significant color due to the presence of bilirubin up to 25 mg. per cent. When 50 mg. per cent of bilirubin, a concentration rarely encountered in serum, was added to a serum containing 5.4 per cent protein, an apparent protein value of 5.5 per cent was obtained. Correction by means of a blank containing the added bilirubin and biuret reagent gave a value of 5.3 per cent; this indicates that the preparation of a blank to correct for the presence of bilirubin is unnecessary.

Slight hemolysis can be tolerated, but excessive hemolysis may give rise to errors. Attempts to correct for excessive hemolysis by means of a blank containing serum diluted with saline have been unsuccessful.

## RESULTS

Excellent agreement is shown by the biuret and Kjeldahl methods when protein determinations are made on normal serum. The results of protein fractionation by the biuret and Kjeldahl methods of blood serums of 6 normal women and 9 normal men are presented in Table II. Kjeldahl determinations were made on 1 e.e. samples of serum. Boric acid was used in the distillation of the ammonia.<sup>5</sup> It is apparent that the accuracy of the biuret method does not differ significantly from that of the Kjeldahl method. In chronic liver disease dif-

ferences averaging 0.5 Gm. have been observed between the results of serum total protein determinations by biuret and Kjeldahl methods.<sup>6</sup> Further studies (to be published) also have shown small differences in some other diseases. It is debatable, of course, whether such differences are due to variation in total nitrogen, in biuret linkages, or both and consequently as to which method is giving results most nearly indicative of the actual concentration of serum protein.

TABLE II

DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS IN NORMAL PERSONS\*

| DATE           | SEX | BIURET                        |                               |                                |        | DIFFERENCE (KJELDAHL MINUS BIURET) |                               |                                |       |
|----------------|-----|-------------------------------|-------------------------------|--------------------------------|--------|------------------------------------|-------------------------------|--------------------------------|-------|
|                |     | T.P.<br>(GM. PER<br>100 C.C.) | ALB.<br>(GM. PER<br>100 C.C.) | GLOB.<br>(GM. PER<br>100 C.C.) | A/G    | T.P.<br>(GM. PER<br>100 C.C.)      | ALB.<br>(GM. PER<br>100 C.C.) | GLOB.<br>(GM. PER<br>100 C.C.) | A/G   |
| 1/22/41        | F   | 7.1                           | 4.5                           | 2.6                            | 1.7    | 0.2                                | 0.0                           | 0.2                            | -0.1  |
| 1/22/41        | F   | 7.2                           | 5.4                           | 1.8                            | 3.0    | 0.0                                | 0.1                           | -0.1                           | 0.2   |
| 5/16/41        | F   | 6.5                           | 4.7                           | 1.8                            | 2.6    | 0.2                                | -0.1                          | 0.3                            | -0.2  |
| 5/27/41        | F   | 7.9                           | 5.2                           | 2.7                            | 1.9    | 0.2                                | 0.0                           | 0.2                            | -0.1  |
| 5/20/41        | F   | 7.0                           | 5.4                           | 1.6                            | 3.4    | 0.1                                | 0.0                           | 0.1                            | -0.2  |
| 5/20/41        | F   | 7.2                           | 4.4                           | 2.8                            | 1.6    | -0.1                               | 0.0                           | -0.1                           | 0.0   |
| 7/ 1/41        | M   | 7.3                           | 5.5                           | 1.8                            | 3.1    | 0.1                                | -0.1                          | 0.2                            | -0.4  |
| 7/ 1/41        | M   | 7.8                           | 5.2                           | 2.6                            | 2.0    | 0.0                                | 0.2                           | -0.2                           | 0.3   |
| 7/ 1/41        | M   | 8.3                           | 5.9                           | 2.4                            | 2.5    | 0.0                                | 0.0                           | 0.0                            | 0.0   |
| 7/ 8/41        | M   | 7.3                           | 5.6                           | 1.7                            | 3.3    | 0.1                                | 0.1                           | 0.0                            | 0.1   |
| 7/ 8/41        | M   | 7.6                           | 5.8                           | 1.8                            | 3.2    | 0.0                                | 0.0                           | 0.0                            | 0.0   |
| 7/ 8/41        | M   | 7.0                           | 5.5                           | 1.5                            | 3.7    | -0.2                               | -0.1                          | -0.1                           | 0.2   |
| 7/11/41        | M   | 8.1                           | 5.8                           | 2.3                            | 2.5    | -0.2                               | -0.1                          | -0.1                           | 0.1   |
| 1/22/41        | M   | 7.2                           | 5.1                           | 2.1                            | 2.4    | 0.1                                | 0.0                           | 0.1                            | -0.1  |
| 9/16/41        | M   | 6.9                           | 5.2                           | 1.7                            | 3.1    | 0.1                                | -0.1                          | 0.2                            | -0.4  |
| Mean           |     | 7.36                          | 5.28                          | 2.08                           | 2.66   | 0.0±                               | 0.00                          | 0.05                           | -0.0± |
| Standard error |     | ±0.035                        | ±0.023                        | ±0.041                         | ±0.054 |                                    |                               |                                |       |

\*Three to four hours after breakfast.

## SUMMARY

The direct biuret method for the determination of serum total protein and albumin has been modified and improved. Rapid and accurate determinations of serum proteins up to 11 Gm. per 100 c.c. in 0.1 c.c. samples of serum can be made by the new method. Application of the method to visual and photoelectric colorimetry is outlined.

I am indebted to Dr. John G. Reinhold, of the Division of Biochemistry, for suggestions and criticism.

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# MEDICAL ILLUSTRATION

## A SIMPLE METHOD FOR DETERMINATION OF EXPOSURE TIME IN PHOTOMICROGRAPHY\*

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TO THE nonprofessional photographer who does not use photographic equipment often enough to become expert in its manipulation, the simple application of mechanical devices that increase his efficiency is of great help. Photomicrography gives rise to problems which are particularly concerned with the amount of light that reaches the photographic plate. If the specimen being photographed varies in the intensity of the stain in different areas and also in thickness, as in supravital stained tissue mounted *en masse*, it is difficult to determine by visual inspection the exposure time for different fields even in the same specimen. With sufficient experience one can estimate roughly the exposure time, but several photographs are often required to determine the correct time before satisfactory results are obtained. This frequently causes an unnecessary waste of time and materials.

The technique to be described uses standard equipment in a rather simple manner. A photoelectric cell (Fig. 1 A) was built into the wide end of a small camera having a fixed bellows (Fig. 1 B), the narrow end of which consisted of tubing (Fig. 1 C) about one-half inch long that fitted snugly into the microscope barrel in place of the ocular. The photoelectric cell was connected to an exposure meter (Fig. 2),† to record the intensity of light being transmitted through the microscope. Fig. 3 shows the complete equipment.

In using this equipment the field to be photographed is chosen and carefully focused. The microscope ocular is then removed, and the photoelectric cell is put in its place. A light reading is taken on the exposure meter. The photoelectric cell is replaced by the camera, and the focus is checked. The time of the first exposure is guessed. With the same setup additional shorter and longer exposures of the same specimen are made, the variations in time being figured in geometric progression. To economize on both photographic material and time, the trial exposure method,<sup>1</sup> in which several exposures are made on

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†This photoelectric exposure meter P. W. can be obtained from Pfaltz & Bauer, Inc., Empire State Building, New York.

one plate, may be used. In this way one eventually arrives at the correct exposure for a given reading on the exposure meter. This procedure is repeated for different meter readings.

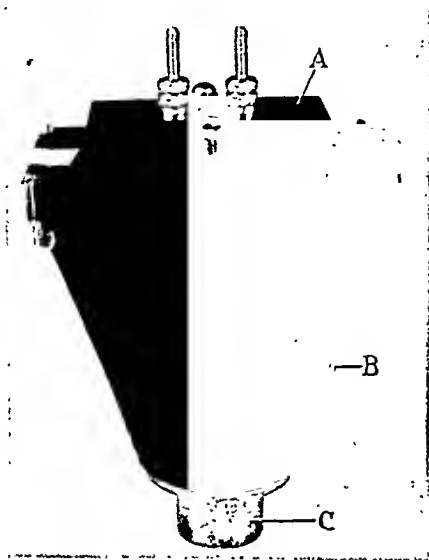


Fig. 1.—Photoelectric cell mounted on camera bellows. A, Photoelectric cell; B, camera; C, adapter for microscope tube.

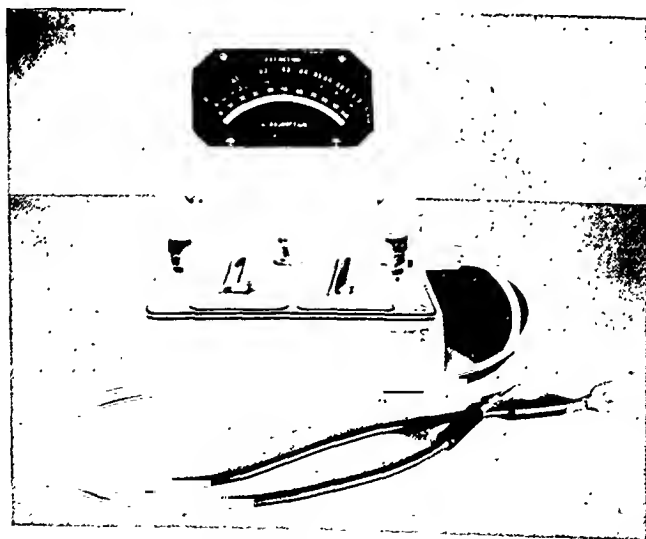


Fig. 2.—Exposure meter.

We used the foregoing method to establish a workable curve (Fig. 4) which would suit our particular purpose. After several successful photographs were made with different amounts of light, we plotted our points, using exposure meter units as the abscissa and the time of exposure as the ordinate. Then we drew a rough curve and joined these points. In order to add intermediate points to our curve and at the same time prove its validity, several additional points along its course were tested. Good negatives were obtained by using the meter

readings and exposure times which fell on the curve. Negatives which were not made with the amount of light, and exposure time indicated by the curve were either overexposed or underexposed.

The curve obtained in Fig. 4 resulted when Wratten M plates and D-76c developer were used. Different types of plates would naturally give different curves.

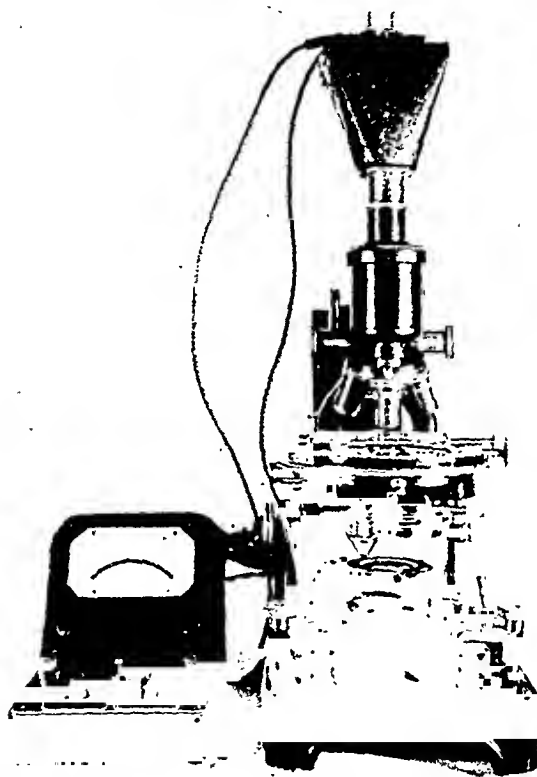


Fig. 3.—Complete equipment assembled with microscope.

Although the curve was constructed chiefly from low-power photomicrographs ( $\times 100$  to  $\times 200$ ), it was tested for higher systems of lenses, including oil immersion, and found to be equally useful. Satisfactory pictures were obtained, regardless of specimen, thickness of section, stain, or magnification. The only requirement to be observed rigidly is that the exposure time be varied according to the exposure meter reading. The meter reading itself can be adjusted by changing the aperture of the diaphragm on the substage condenser, or in the light source if one is available there. This technique permits one to keep the intensity of light within the range of the curve that is most convenient for the exposure time units permitted by the mechanism of the camera shutter.

In Fig. 5 the application of the curve in Fig. 4 is illustrated. Extreme differences in light intensity and in magnification are shown in the four photographs. In A the amount of light employed was small, only 2 meter units, and the exposure time, as determined by the curve, was relatively long, one second.

In *B* the conditions were reversed, for the light meter reading was 55 and the exposure time was  $\frac{1}{25}$  second. In *C* the magnification was low,  $\times 100$ , the light intensity was 15 units, and exposure time was  $\frac{3}{10}$  second; in *D* the magnification

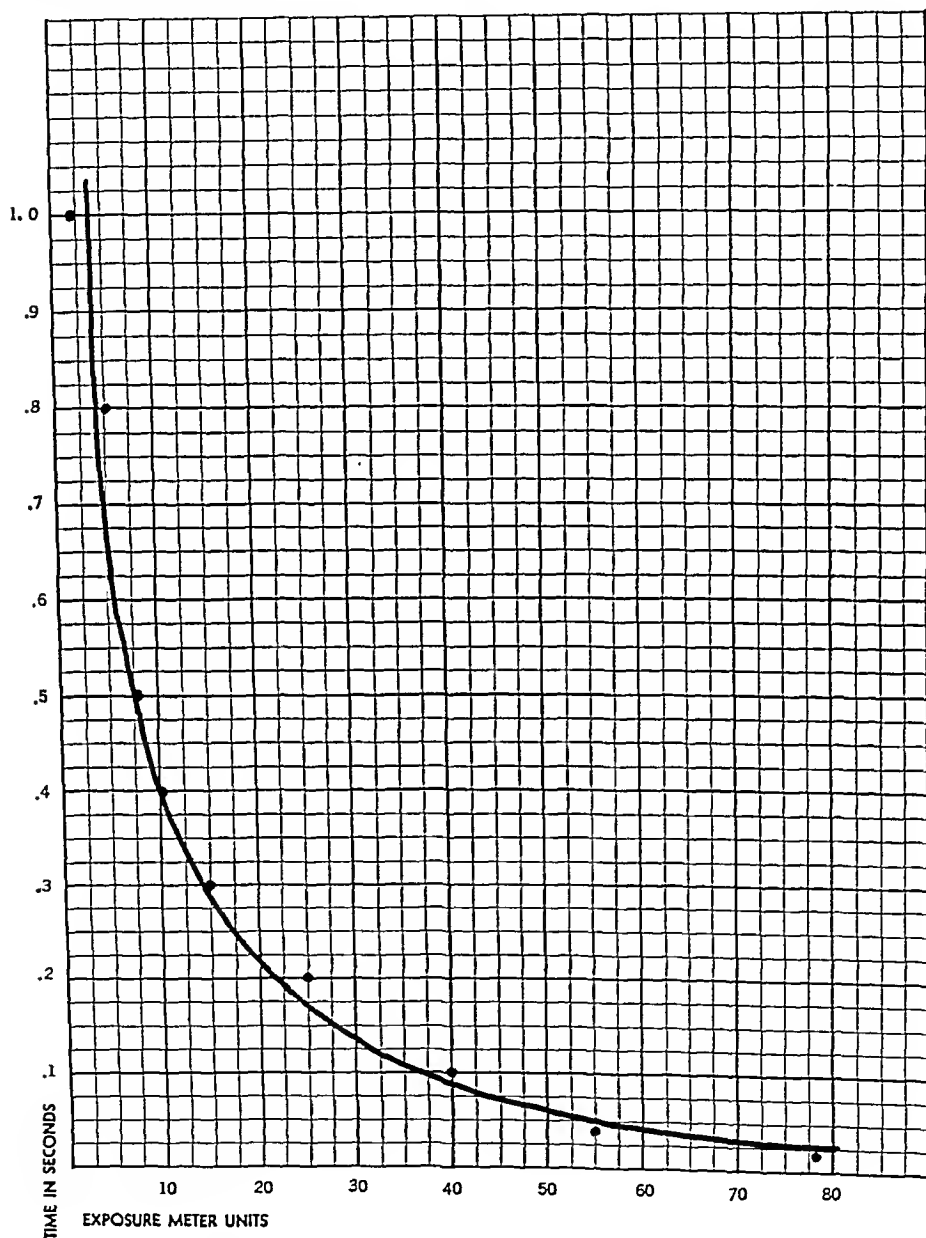


Fig. 4.—Graph showing relation of exposure meter units to exposure time.

was  $\times 900$ , with the light intensity and exposure time the same as in *C*. The development and printing techniques were comparable in all instances. Slight variations in the depth of the final print, as in *A* and *B*, may be adjusted to suit one's particular purpose during the printing process.

When filters that exclude much of the available light are used, the equipment may not be sensitive enough to register the amount of light reaching the photoelectric cell. In such instances it was found advantageous to take exposure meter readings without the filters and to make trial exposures for determining the time with the filters in place. The correct exposure time was naturally much greater for any light meter reading than that recorded in Fig. 4, because the photographs were actually taken with much less light than that used for determining the exposure meter reading. A workable curve, similar to that in Fig. 4, was made for such low light intensities and long exposures.

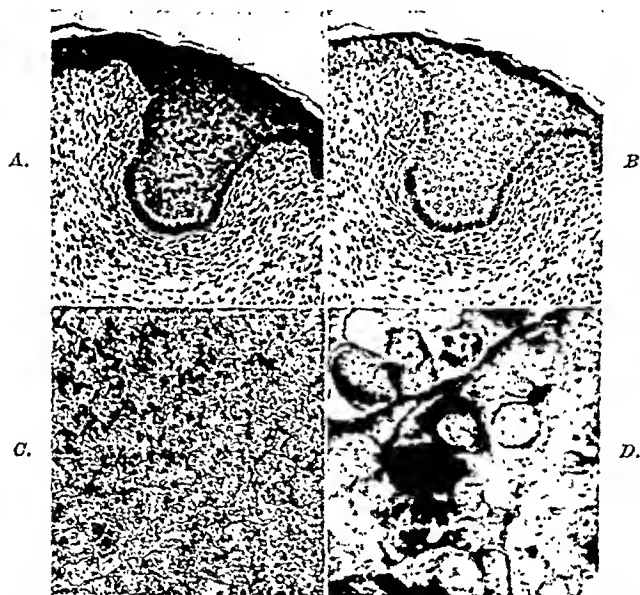


Fig. 5.—Sample photographs obtained by means of the graph illustrated in Fig. 4. The developing and printing processes were the same in each instance. *A*, Mammary gland anlage of treated pouch-young opossum,  $\times 100$ . Exposure meter reading, 3 units; exposure time, 1 second. *B*, Same as in *A*,  $\times 100$ . Exposure meter reading, 55 units; exposure time,  $1/25$  second. *C*, Anterior lobe of hypophysis of guinea pig,  $\times 100$ . Exposure meter reading, 15 units; exposure time,  $3/10$  second. *D*, Same as *C*,  $\times 900$ . Exposure meter reading, 15 units; exposure time,  $3/10$  second.

#### SUMMARY

A simple method for determining the exposure time in photomicrography by the use of a photoelectric cell and exposure meter is described.

We wish to express our appreciation to Professor Eduard Uhlenhuth for his interest and advice in working out this problem. We wish to give full credit to Doctors Raymond K. Thompson and Glenn H. Algire, who adapted the photoelectric cell as described in this paper.

#### REFERENCE

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# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**PERTUSSIS**, a Note on the Cultivation of *Hemophilus*, Anderson P. M. M. J. Australia 2: 224, 1941

Flexible copper wire, gauge 19, was used in lengths of 15 cm., and on one end a tiny loop was made and dressed with a thin layer of cotton wool. These swabs were sterilized in tubes and were then ready for use. For the taking of the swab the child was seated on its mother's lap and the head was extended firmly against her shoulder. The manipulator bent over the child and passed the wire along the wider anterior nares into the post-nasal space. A side-to-side movement was then imparted to the swab, and the finger could feel the tip passing over the posterior pharyngeal wall. It was then quickly withdrawn, the end of the swab being depressed slightly and describing an arc. The wire was usually found to have taken on a curved shape.

Swabs were taken from children as young as 3 months old, and no difficulty was encountered; toddlers and older children occasionally coughed, but no failures occurred. The swabs were rubbed onto Bordet-Gengou medium containing 15 per cent of horse blood and incubated for four days, and a satisfactory proportion of positive cultures was obtained.

**McCLURE-ALDRICH TEST.** A Clinical Study and Evaluation, Lichtenburg, N. H. A. J. Dis. Child. 62: 743, 1941.

Thirty-three newborn infants given the McClure-Aldrich test on the first day of life and 50 infants tested after the "physiologic weight loss" was corrected had an average saline absorption time of 10.4 minutes.

Seventy normal newborn infants who were given no supplementary fluids and were tested at the time of greatest "physiologic weight loss" had an average saline absorption time of 26.3 minutes, two and a half times as long as that of the control groups.

Twelve normal newborn infants who had an average saline absorption time of 9.9 minutes on the first day of life showed an increase of absorption time to an average of 20.8 minutes on the third and fourth days, when the weight loss was maximum. The absorption time returned to an average level of 12.7 minutes on the day of discharge, when the weight loss and the state of hydration were, in part at least, corrected.

There was no difference between the absorption times of physiologic solution of sodium chloride and a solution of twice that concentration. Normal potassium chloride solution, however, was absorbed more than twice as quickly as physiologic solution of sodium chloride.

The McClure-Aldrich test is valuable in determining and following up the state of hydration of the body tissues. The absorption time depends, however, on the state of hydration at the site at which the test is performed and the mechanical ease of diffusibility of the injected saline solution into the intercellular spaces of the skin. It may be true that edema is a result of local or generalized toxicity and anoxemia and represents an attempt by the body to dilute the toxins. The result of the McClure-Aldrich test, however, may be regarded as indicative only of the extent and intensity of the edema, regardless of its cause. It may be a measure of tissue toxicity but only so far as, and when, the toxicity directly parallels the local edema.

**NEPHROTIC SYNDROME**, Acacia in the Treatment of, Goudsmit, A., Jr., Binger, M. W., and Power, M. H. Arch. Int. Med. 68: 701, 1941.

In 28 patients with the nephrotic syndrome who were treated with acacia the following observations were made:

1. The concentration of acacia in the serum resulting from the injection of 60 to 150 Gm. ranged between 1,000 and 2,500 mg. per 100 c.c.

2. The concentration of serum proteins usually diminished. As an average this amounted to 22 per cent of the original concentration. This related decrease could not be correlated with the amount of acacia administered, with the resultant concentration of acacia in the serum, or with the initial concentration of serum proteins. Increases in circulating plasma volume and concomitant dilution of its constituents appear to account adequately for the decrease of serum proteins.

3. The gross changes in colloid osmotic pressure subsequent to injections of solution of acacia are directly related to the absolute changes of the concentration of the serum proteins and of the concentration of acacia. If certain values are assigned for the "specific" osmotic pressures of serum proteins and of acacia, the changes of colloid osmotic pressure can be predicted fairly accurately. In approximately half of the patients the calculated changes were within 6 mm. of the observed values.

4. The colloid osmotic pressure of the serum may be increased, unchanged, or decreased after a course of injections of solution of acacia. An increase is most likely to occur when the initial colloid osmotic pressure is most subnormal.

5. These changes of colloid osmotic pressure, under the general regimen to which these patients were exposed, appear to be essentially unrelated to the degree of effectiveness of the treatment.

**SULFANILAMIDE, A Simple Micro Test for, and Its Derivatives in Blood, Churg, J., and Lehr D. Am. J. M. Sc. 202: 589, 1941.**

#### REAGENTS

A. 15 per cent aqueous solution of trichloroacetic acid.

B. 2 per cent para-dimethylaminobenzaldehyde (p-BA) in 95 per cent ethyl alcohol. (If stored in a glass-stoppered brown bottle, the reagent keeps for at least a month. It is almost colorless if prepared from fresh p-BA. Crystalline substance which has turned strongly yellow due to prolonged standing is unsuitable for use.)

C. N/6 HCl (for determination of total sulfanilamide concentration).

#### EQUIPMENT

A. Test tubes, 10 mm. in diameter, calibrated at 1.5 c.c., 2 c.c., 3 c.c., and 4 c.c., with rubber stoppers.

B. Blood pipette, 0.1 c.c.

C. Small (1 inch) funnel and filter paper (Whatman No. 2, 4.25 cm.).

D. Graduated centrifuge tubes (for determination of total sulfanilamide concentration).

E. Comparator (block or slide) with blue glass filter, a set of permanent standards (Table I) and comparison tubes (10 mm. inside diameter) calibrated at 1 and 1.5 c.c.

TABLE I  
COMPOSITION OF PERMANENT NONFADING STANDARDS

| K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub><br>(C.C.) | K <sub>2</sub> Cr <sub>2</sub> O <sub>4</sub><br>(C.C.) | H <sub>2</sub> O<br>(C.C.) | CORRESPONDING CONCENTRATIONS<br>OF SULFANILAMIDE IN BLOOD<br>(MG. IN 100 C.C.) |
|---|---|----------------------------|--|
| 0.5   | 0.1   | 9.4                        | 0  |
| 1.0   | 0.25  | 8.75                       | 1  |
| 1.5   | 0.4   | 8.1                        | 2  |
| 1.9   | 0.5   | 7.6                        | 3  |
| 2.2   | 0.7   | 7.1                        | 4  |
| 2.5   | 0.9   | 6.6                        | 5  |
| 2.7   | 1.3   | 6.0                        | 6  |
| 3.0   | 2.1   | 4.9                        | 8  |
| 3.0   | 3.7   | 3.3                        | 10   |
|   | 0.5%  |                            |  |
| 3.0   | 5.0   | 2.0                        | 15   |

## PROCEDURE

*A. Determination of Free Sulfanilamide*

1. Place 1.5 c.c. of water in the calibrated test tube.
2. Add 0.1 c.c. of blood (drawn from finger or ear lobe) and rinse the blood pipette several times with the water in the tube. Shake the tube gently for a minute or two until the blood is completely hemolyzed.
3. To precipitate the proteins, add 15 per cent trichloroacetic acid up to the 2 c.c. mark (approximately 8 drops). Close the tube with a rubber stopper and invert several times. This step may be performed immediately following 2, or within several hours.
4. After two minutes filter the mixture directly into the comparison tube up to the 1 c.c. mark. Instead of filtering, the mixture in the precipitation tube may be centrifuged for five minutes at high speed, and 1 c.c. of clear supernatant fluid transferred to the comparison tube by means of a capillary pipette. This is especially convenient if several tests are run simultaneously.
5. Add the p-BA up to the 1.5 c.c. mark, mix by inversion and compare with the standards in the comparator, either immediately or within two hours. In the latter case the comparison tube must be tightly stoppered.

The p-BA reagent should be shaken before use.

If the drug level in the blood is found to exceed 10 mg. per 100 c.c., accurate determinations are obtained by repeating the test with double dilution of the blood. For that purpose 0.1 c.c. of blood is hemolyzed in 3 c.c. of water and trichloroacetic acid added up to the 4 c.c. mark (about 18 drops). Otherwise the procedure remains unchanged. The readings are multiplied by 2.

*B. Determination of Total Sulfanilamide*

The procedure as described above remains unchanged up to Step 4. Steps 4 and 5 are modified as follows:

4. Place 1 c.c. of the filtrate or centrifugate in a graduated centrifuge tube, add 1 c.c. of N/6 HCl, mix, and heat in a boiling water bath for one hour in order to hydrolyze the acetylated sulfanilamide. The mixture evaporates almost completely. Cool to room temperature and bring the volume up to 1 c.c. with water.
5. Add the p-BA reagent up to the 1.5 c.c. mark, mix by inversion, transfer into a comparison tube and compare with the standards. The readings are then multiplied by 1.15 to compensate for the decrease in color intensity due to the addition of hydrochloric acid.

*C. Determination of Sulfanilamide Derivatives*

With appropriate standards the test can be applied equally well to the determination of sulfapyridine, sulfathiazole, and other sulfanilamide derivatives. For clinical purposes, sufficiently accurate results can be obtained with these compounds by using the sulfanilamide standards for comparison, provided the readings are multiplied by the following factors:

|               | Free | Total |
|---------------|------|-------|
| Sulfapyridine | 1.5  | 1.7   |
| Sulfathiazole | 1.7  | 2.0   |

These factors take into account the higher molecular weight of sulfapyridine and sulfathiazole, the loss in precipitation due to the poor solubility of these compounds, and, in determination of total drug concentration, also the decrease in color intensity caused by the addition of hydrochloric acid.

**T. N. T. Derivative in Urine, Improved Webster Test for, Ingham, J.** *Lancet* 2: 554, 1941.

Place 50 c.c. of urine in a convenient sized beaker (400 c.c.) and add 50 c.c. of 20 per cent (by volume) sulfuric acid (1 part concentrated  $\text{H}_2\text{SO}_4$  plus 4 parts  $\text{H}_2\text{O}$ ). Mix.

Place beaker on tripod, supported by wire gauze, and heat over Buusen flame till the liquid boils.

Boil for one minute; allow to cool. Cooling may be hastened by running cold water over outside of beaker.

After cooling, pour the mixture of urine and acid into a separatory funnel of 200 c.c. capacity and add 50 c.c. of ordinary (methylated) ether. Shake up well and then allow to settle for about fifteen minutes. The lower layer of urine and sulfuric acid is next run off by removing the top stopper and opening the lower tap.

The ethereal solution of acidified urine remaining in the separatory funnel is then washed twice with 1 per cent aqueous solution of sodium carbonate. This is done as follows. Add 20 to 25 c.c. 1 per cent sodium carbonate (aqueous), shake, and, after allowing to settle for five to ten minutes, run off the lower layer. Repeat. (If the person has been taking purgatives, such as rhubarb, senna, cascara, phenolphthalein, which yield anthraquinone derivatives, the sodium carbonate solution will show a reddish-violet color).

After this treatment with sodium carbonate the ethereal solution in the funnel is washed with water, as follows. Add 50 c.c. tap water, shake, allow to settle for fifteen minutes, and then run off the lower layer as before. (Occasionally a troublesome emulsion forms in the ethereal extract while washing with water but it is easily dispersed by adding a few drops of absolute alcohol and shaking gently.)

Now place 5 c.c. of the ethereal extract, so washed and prepared, in a test tube and quickly add, without shaking, 5 c.c. of a 5 per cent alcoholic solution of potassium hydroxide (5 gr. caustic potash dissolved in 100 c.c. of methylated spirit of absolute alcohol). A violet cloud indicates a positive result. The liquid in the test tube should be watched carefully, for the color develops quickly and just as quickly fades. The intensity of the color varies from the faintest tint to a deep violet according to the amount of TNT in the urine.

The unused ethereal extract remaining in the separatory funnel need not be thrown away but may be collected and later separated off from any TNT by distillation. It may then be used again in other tests.

For the routine everyday urine examination of workers in TNT factories the technique may be simplified as follows:

*Apparatus Required.*—Two ordinary test tubes (7 inches by  $\frac{3}{4}$  inches); Bunsen burner or spirit lamp.

*Reagents Required.*—20 per cent (by volume) sulfuric acid; ordinary (methylated) ether; 5 per cent alcoholic solution of potassium hydroxide (freshly prepared).

Place about 10 c.c. of urine in one test tube; add about an equal quantity of the 20 per cent sulfuric acid; shake and bring to a boil; cool thoroughly under water tap.

*When cold*, add about 10 c.c. of the ether and shake well; allow to stand a few minutes until ether floats to the top. Pour some of the supernatant ether into the other test tube and to it add about 2 c.c. of the 5 per cent alcoholic potassium hydroxide solution. A dirty reddish purple color develops at once if TNT is present. The tint varies from faint to very deep according to the amount of TNT present and appears quickly and just as quickly fades. If no TNT is present, a light brown color only results and persists.

With this simplified technique the test is sensitive to 1 part in 10,000. It does not exclude anthraquinone derivatives (from the purgatives mentioned) which give a similar but pinker color which persists.

### Erratum

On page 334 of the December issue of the JOURNAL in an article entitled "Age Incidence of Positive Tuberculin Reactions (Mantoux)" by Frances Pascher, M.D., and Marion B. Sulzberger, M.D. (with the statistical analysis by Alexander S. Wiener, M.D.), the formula appearing on the last line of the page should read:

$$P.E._{DIFF.} = \sqrt{(P.E._{P_1})^2 + (P.E._{P_2})^2}$$

Also on page 335 of the same article the fourth line under Table III should read:

"Therefore,  $p_2 - p_1 = 29.5$  per cent and  $P.E. (p_2 - p_1) = \sqrt{(0.06)^2 + (0.051)^2} = 0.081$ , or 8.1 per cent."

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GEORGES HAYEM (1841-1935)

*"The future belongs to hematology."—Georges Hayem*

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IT IS A great honor for me to speak before your esteemed Society in commemoration of the hundredth anniversary of a countryman who is called the father of hematology. His anniversary will not be celebrated in his fatherland, where an atmosphere of constraint and persecution compels the best of our profession to a humiliating silence. There it will be impossible to praise Georges Hayem despite all his contributions to the glory of his country. I am thus doubly fortunate to be able to express to you my deep feeling of gratitude and appreciation of your hospitality. I should also like to offer my very good friend, Dr. Nathan Rosenthal, my sincere thanks for this opportunity to commemorate Georges Hayem.

When, as a young doctor, I went to Paris for the first time, I attended a meeting of the Société médicale des hôpitaux de Paris on the Rue de Seine. Seated beside me was a little old man, his tanned face crowned with a snow white crop of abundant hair, with locks falling to his neck. He had a stiff little beard, a snub nose surmounted by gold spectacles; a vivid and piercing eye completed this remarkable head of a true scientist. I was deeply impressed by his appearance, and it was not until later that I learned that this old man, who was treated with great respect and deference, was Georges Hayem.

The story of his life hardly lends itself to the dramatics of a romanticized life. It is earnest and straight as the life of so many seekers after truth, and it seems that he experienced his allotment of great emotions, sudden turns of fortune, joys, and disappointments in the laboratory, when a hypothesis became a truth, or when a new observation came to overthrow a truth of yesterday. But we are not qualified to judge. Under a calm and impenetrable mask sometimes unfolds a tormented existence; and a life of action is often but an escape from

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personal suffering. I do not wish to have you believe for a moment that such was the case of Georges Hayem. However, I wish to indicate how frequent it is for people to take the secrets of their inner lives to their graves. Nevertheless in his life, it is obvious that there is nothing to remove it from the framework of a normal life.

He was born in Paris, Nov. 24, 1841, the son of Simon Hayem, a successful businessman of the Sentier section, who had hoped that his son would succeed him. By some strange fate, Georges Hayem preferred to be a student, and, perhaps by atavism, to choose a medical career.

His family had preserved the portrait and the memory of an ancestor, Isaie Servus Ulman, who practiced medicine in Metz. Louis XV, at that time still called Louis the Well-Beloved, having accompanied the army in the War of the Polish Succession, was seized in Metz with a bad case of dysentery. As the condition of the prince was growing progressively worse, the royal physicians, overcoming their prejudice, in desperation called in the Jewish doctor. Against every expectation, the remedies of Isaie Servus Ulman brought about a cure. In the midst of the delirious joy ushered in by this news to the entire kingdom, Hayem's ancestor deemed himself happy to obtain as his reward an improvement in the conditions of the Jews of Lorraine. There is no doubt that such a family heirloom was largely responsible for directing young Hayem toward medicine.

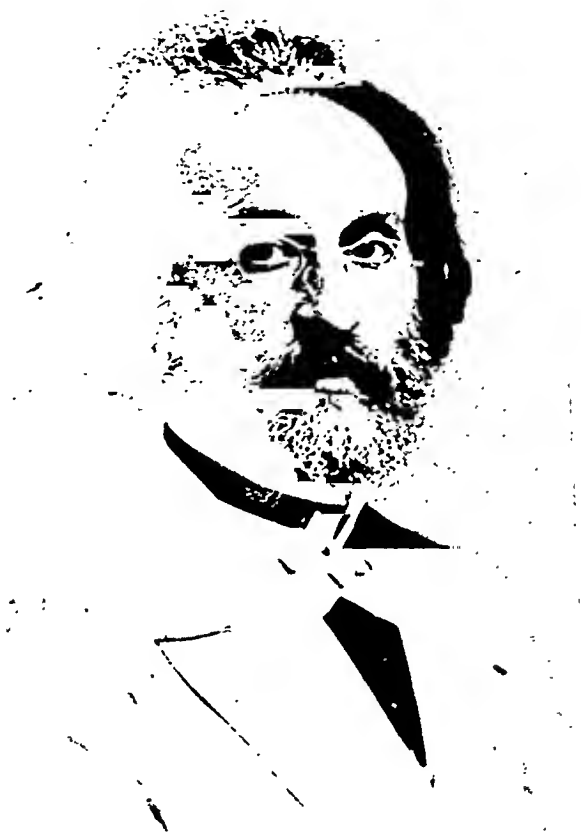
Georges began his medical studies in 1861. He completed a two-year course in one year; this was a remarkable achievement. In 1862 he received the highest honor in the examination for externship. He was admitted as an interne in 1863. He received the gold medal in 1867 from the Académie de médecine for his brilliant work. In 1872 he was accepted as a physician in the hospitals and was licensed to practice. Seven years later he was named titular professor, and finally in 1886 he was elected to the Academy of Medicine. He was just 45 years old.

The exceptionally rapid ascent cost Hayem many jealousies and grudges. But he was not the man to bother with public opinion. He was sure of himself, conscious of his worth, and seldom, if ever, stepped aside from this line of conduct. Henceforth, his life unfolds between his service at the hospitals and at home. He used to arrive early in the morning at the Saint Antoine in a fiacre. On entering his office he donned a simple white apron and a black velvet skull cap for ward rounds. He did not waste time in the exchange of civilities with his co-workers. He was rough and brisk and demanded much from others, as well as from himself. The students did not love him; they feared him. Much courage was needed to become his interne.

Paul Bensaude told me to what extent Hayem was feared. His brusqueness at first repulsed people, but at bottom this rudeness concealed timidity, and his closest associates knew that under this façade of brusqueness beat a heart on which one could depend. The fidelity they gave him is a touching testimony of their comprehension of this great man.

The public liked him. The people of the Saint-Antoine district greeted him with respect. His fame dates from the epidemic of cholera which ravaged Paris in 1870, during the siege. Hayem did not spare himself in caring devotedly and courageously for the stricken. He noticed that a great number of

the cholera victims died of dehydration. By intravenous injection of artificial serum, isotonic saline, or Hayem's serum, he managed to save 30 per cent of his patients, a considerable number for the time. This success brought him his nickname, Doctor Cholera.



Georges Hayem.

Until his death Hayem remained the master who looks upon his associates as his disciples. Bensaude, of whose world-wide renown by virtue of his work in proctology I need not remind you, has, like many others, surrounded Hayem with filial devotion. A few days before his death, Hayem asked for a camphor injection, and requested Bensaude to perform it. Hayem thanked him, with this remark: "Bensaude, you still don't know how to give an injection." Bensaude was then 67 years old.

In 1911, caught by the age limit, Hayem had to quit service at the Saint-Antoine Hospital. In the course of his farewell address, at the moment when he must leave his laboratory and his associates, you perceive the soft murmur of this sad life of the scientist whom others, more lucky than himself, have surpassed:

"I cannot complain," he said, "I have studied and taught medicine during a half century which shone with incomparable luster: the work of Pasteur, the

ascent of surgery, the discovery of serotherapy and radiology. This incessant, tumultuous growth of knowledge, ringing discoveries, is comparable to a rising tide that keeps on rising. How small one feels during the submersion! How fitting it is to be humble!"

In his private life cruel experiences were in store for him. First he lost a daughter whom he adored. A terrible malady overtook his wife. His older son was killed at Verdun. Later he lost his second son. To withstand implacable destiny, only one daughter was left him, and the affection of his son-in-law, his grandchildren, and his pupils.

On the sixth floor of his house at Auteuil, Hayem furnished a workroom where, in his last years, he practiced a hobby, modeled medallions, and showed his skill at painting.

It is impossible in the short time allotted me, to pass in review all the scientific work of Georges Hayem. His publications are so numerous and diverse that we are forced to limit ourselves to hematology. But we must not forget that other fields, particularly that of gastroenterology, were strongly influenced by his research.

Before launching on our selected subject let us mention, however, that his thesis for the doctorate was entitled "Studies on Diverse Forms of Encephalism" (1868), and that of his two master's theses, one treats of "Bronchitis" and the other of "Arachnoid Hemorrhages" (1872).

What was hematology in France before Georges Hayem? This question has not until the present had the development it merits. To discuss this question one must first descend to the obscure sources of modern medicine. Let us refer to two works, rarely cited, but which constitute in my mind the milestones on the narrow path leading out on the wide road of Georges Hayem's hematology. The first work is entitled "The Medical Analysis of Blood," and was published by Theophile de Borden in 1775. Only a pamphlet written in the style of the eighteenth century, it constitutes a brief opposing the pretensions of physics and chemistry to invade the field of medicine and to attempt to dominate it. He does not admit that these accessory sciences submit the phenomena of life to their close scrutiny. He speaks of a certain group of physicians "who claim that blood is composed of globules which can be counted, but no one must object to the arbitrary count. They go so far as to imagine bursting globules, broken globules, globules sticking together, as occurs with glass globules."

And, for our edification and consolation, he adds: "Sensible men will not pay attention to this childish prattle." Thus speaks the eighteenth century, opposing with its ironic verve the destiny of medicine. This is routine, mocked by Molière and Montesquieu, which would hold back the advent of a new era.

The second work which will help us form an idea of medicine before Hayem is entitled "An Essay in Pathological Hematology." It was written by Gabriel Andral, Professor of General Pathology and Therapy at the Paris Medical School, Member of the Royal Academy of Medicine, and Staff Member of the Hôpital de la Charité. It was published in 1843 by Fortin, Masson et Cie.

This little treatise seems to me to be of considerable interest in the history of hematology. Here we find in a nutshell the opinion of the period of transition from the eighteenth century medicine to the medicine of Georges Hayem.



The ideas exposed in this booklet surprise us as much as they charm us. And, if Hayem is the Father of Hematology, permit me to name Andral the Grandfather.

I quote for you a few passages of this pioneer's work:

"Until the present, direct observation of the blood has been invoked more rarely than reasoning. To cope with this problem, blood must be analyzed. Hematology will be in a position to gain valuable facts only when the blood of a great number of sick people will be submitted to a chemical investigation and examined under the microscope.

". . . but in order that microscopic analysis of blood might yield truly useful results, one condition is indispensable: namely, that we first acquire an exact knowledge of the varieties of the physiology of blood.

". . . Because of our failure to make a preliminary examination of the physiology of blood, many erroneous assertions have been made in connection with the changes produced in the blood corpuscles by illness. I am not afraid to state that until the present no alteration in form or texture, which might be considered the result of the influence of a disease, was definitely found in these small bodies, and also that all modifications of aspect described and other modifications which were briefly mentioned result from the progressive destruction which they undergo in proportion to the length of time they are cut off from the influence of life."

But aside from these considerations on methods, Andral had ideas on diseases of the blood. His notion, for example:

". . . and it is, in effect, the great increase in number of the red corpuscles which establishes in the blood the characteristics of plethora."

Or this one: "The blood cannot be deprived of a certain quantity of its corpuscles without causing serious disturbances of the nervous system, which are translated by various disorders of intelligence, emotion and movement."

And he adds what we understood only a century later:

"If the facts on this order were submitted to proper verification, they would doubtless prove of extreme importance, since they would demonstrate that the nervous system exerts a great influence upon the constitution of the blood, and consequently, in the same way, the blood, when it is altered, can modify nervous action. Thus, a disturbance of the nervous system is capable of altering the blood."

These sentences, which might have been written in our time, are the more remarkable when we consider that Gabriel Andral had to struggle against his elders, who, he says, "rejected the microscope as useless, or feared it as a source of error."

Only a century ago there was a slogan of the "microscopic illusion," a slogan which risked to push back for years the coming of the scientific era of medicine.

In his textbook *Cours de microscopie*, Alfred Donné explains in a limpid style the struggle he had to sustain to give the indispensable basis to medical sciences in general, to hematology in particular. Here is what we read—not without emotion—in his book published in 1844:

"For seven years I gave myself up wholly to public teaching of microscopy applied to medical studies. I founded this teaching at my cost, at my risks and perils.

"I can draw out satisfaction from my effort since it took place in the midst of the decadence of teaching, despite the indifference of the physicians and the obstacles thrown to the way by microscopic observation itself. The success obtained went beyond what my zeal and my convictions permitted to hope."

And with a just pride he added:

"My lectures, attended by a great number of French and foreign pupils, contributed to make the importance of the microscope understood and to win the interest and the confidence which it merits. I know what there is still to accomplish in diffusing the knowledge of the use of this instrument in medical practice, to popularize it, as it has to be. But by looking back to a half score of years, by seeing the progress of this science, it is impossible to lack confidence in its future."

"I applied," he said, "the instrument to medical studies, the instrument, which was used habitually by botanists and entomologists."

The work of this great and modest man, which an ungrateful forgetting leaves covered with a thick layer of dust in many libraries, merits to be remembered by those who care for the becoming of the sciences. He was the first, I am sure, who performed the idea of projecting the microscopic pictures by means of an apparatus which he constructed himself to allow his pupils to convince themselves of the reality of a world which the majority of the physicians of his time considered as an illusion.

I found in the *Collection of Reports on the Progress of Literature and Science*, published under the auspices of the Ministry of Public Education and edited in 1867 by the Imperial Printing Office, the echo of the memory of Gabriel Andral:

"We must point out again as one of the titles of which French medicine of our day may boast, the research in the field of liquid composition of the diseased organism. The appearance of the small volume entitled 'An Essay in Pathological Hematology' was a real event. This book . . . . has definitely banished a secular error and enthroned a great truth. It was not a question of admitting wherever possible alterations in the blood, but of demonstrating such changes, whenever they occurred."

I realize that I have given much space to Theophile de Bordeu, Gabriel Andral, and Alfred Donné, but I think that their ideas shed more light than could a commentator upon the birth of hematology, upon the "how" environment created by the French clinic, which will permit a mind like Hayem's to perfect that which his predecessors could only hope.

Is it not marvelous to see how these men of random means, in a conservative, even hostile environment, were able, by virtue of their courage and perseverance, to conquer a field, fertilize it, and sow the seed. The harvest, in the work of Georges Hayem, proved beautiful and rich.

The first findings of Hayem came to light at the time Louis Pasteur revealed to the world his discoveries, demonstrating the reality of infections

and identifying microbes. It was the greatest event, the sensation of the century. As a corollary to this discovery, his collaborators studied the organism's defenses in the struggle against the microbe invasion. The function of the white corpuscles was discovered, and it was not at all surprising that they usurped the public interest.

It was at this time that Hayem, somewhat apart from the fashion of the day, set out to build, stone by stone, the magnificent edifice of the red corpuscle. And his great work, "Of Blood and Its Anatomical Alterations," which appeared in 1889, and which he proudly called "long original memoir," will constitute for future generations a monument to contemplate with admiration. In 1878 he had already collected his hematologic publications in a monograph which may be considered as the first textbook on diseases of the blood.

In numerous publications Hayem describes the morphology of the red corpuscle, normal and pathologic. He distinguishes between the artefacts of preparation and pathologic modifications. He created the idea of the color index. He discovered the third figurative element of blood, namely, the platelets; he demonstrated their role in coagulation, a function which was fallaciously attributed to the leucocytes. With marvelous clarity and precision, he explained the phenomenon of coagulation. He showed what profit could be derived from a study of coagulation for the differentiation of hemorrhagic diseases. He taught the notion of the prolonged bleeding time, and observed the retractability of the coagulated blood corpuscle and its nonretractability in certain conditions. He prepared a solution, which to this day bears his name; this solution permits the preservation of the red blood cells without altering them for counting. After Potain and Malassez, he improved the hematologic technique. The list could be indefinitely extended, if I were not afraid of overstepping the limit of time granted me. At every instant, in the practice of treating diseases of the blood, we draw on his experience; at every step we utilize what we inherited from Hayem.

But this heritage became classical and, therefore, anonymous. Many valuable procedures in hematology were introduced by Hayem, but we have forgotten their origin as we have forgotten who taught us to walk.

Aside from these questions of technique, physiology, morphology, we owe to Hayem clinical descriptions of hematologic disorders, which have retained their full value throughout years of progress. It is revealing to read with what care he took, and made others take, his clinical observations in order to find an explanation for them. We who walk over an explored earth can hardly conceive of the difficulties and obstacles on the path of endeavor that this pioneer Hayem assigned himself.

With an admirable clinical sense he treads his way through the hemorrhagic diseases. The hematology of purpura is almost entirely his work. He presents this nosologic group with exemplary clarity:

"1. Scurvy, characterized by etiological conditions in which it is observed. It is found among individuals who were subjected to an inadequate diet . . . by the absence of certain essentials, and particularly those contained in vegetables.

"2. Hemophilia, a hereditary disease, characterized by a considerable diminution of the coagulability of the blood, that is, the property of the blood to join in a mass outside the organism; and by producing abundant hemorrhages on the occasion of the slightest wound.

"3. Purpura, 'a symptom and not a clinical entity.' The characteristics of purpura hemorrhagica from the hematological point of view are:

"a. The absence of appreciable anatomical modifications in the red corpuscles.

"b. A considerable diminution of the number of hemoblasts or platelets.

"e. The lack of constant modifications of the white corpuscles.

"d. Normal coagulation time of the blood.

"c. Absence of the transudation of the serum, resulting from the loss of the property of contraction, which the normal clot possesses." (*Lessons on the Diseases of the Blood*, 1900, page 532.) Later (page 588) Hayem complete this table with another sign: the prolongation of the bleeding time. Who would have guessed, I ask you, that his notations were written a half century ago?

In order to explain the pathogenesis of the purpura hemorrhagica, he recalls his experimental observations, collected at the time of injecting the serum of a bull into a dog. "The symptoms," he writes, "that occur in dogs thus treated correspond exactly to those of the acute hemorrhagic purpura."

And he wonders whether it is possible that "under the influence of certain infections, toxic infections, or even simple auto-intoxications or disturbances of the general diet, alterations in the serum appear, similar to those that are brought about by the heterogeneous serum."

You will perhaps reproach me for having quoted too much. But, in submitting to you all these texts, I wished to have you share in my pleasure and admiration for the luminous and proverbially French clarity of the master. Besides, there is a pitfall to be avoided:

The ideas of today, as of all times, are impregnated and supported by aspirations, by notions barely formulated, common to our contemporaries, which create the atmosphere of a generation. Thus, all too often, in reading or commenting the work of a forerunner, we have the tendency to introduce into the thought of that author our own background, the subconscious plan of our own ideas; we let ourselves go, illuminating with today's light the landscape of Yesterday's Master.

"An ardent reader," wrote Michel de Montaigne, "often discovers in the writings of others perfections which the author had not accomplished or even noticed."

In order to avoid the risk of an interpretation of this type I have let Georges Hayem speak for himself. You are the judges.

The importance of an author, said our André Gide, is contained not only in the value of his work, but also in the opportunity of his message. Georges Hayem's message was certainly timely. Andral was still maintaining that blood was nothing but "liquid flesh." But Hayem knew that to describe the diseases of the blood, he had to describe the diseases of the hematopoietic system. Here is the grand idea formulated: "Very often," he says, "there are present in anemia cases, sensitive modifications of the hematopoietic system, and not merely functional disturbances."

The framework of anemia which he traces has lost nothing of its actuality.

"1. Diseases caused by hematopoietic disturbances, characterized essentially by anemia.

"2. Anemias caused by loss of blood.

"3. Diseases resulting from a hyperplastic or neoplastic lesion of the hematopoietic organs. Leukemia and pseudoleukemia.

"Toxemia. Saturnine Anemia."

One finds in the body of his memoir, as he calls it, ideas which not only answer the needs of his own time, but which also constitute as Montaigne remarks, "food likely to nourish divers hungers of successive generations."

This one for example:

"Blood and serum possess specific properties as to species, the idea of the species being all-important; it will be painful to see it substituted by the idea of toxicity."

Or this idea, expressed in a discussion of the nature of pernicious anemia:

"Pernicious anemia . . . is anemia in those who, while taking the necessary food, have become incapable of assimilating it and extracting from it the necessary materials for the reparation of the blood."

Or this one: "The presence of red corpuscles with nuclei will facilitate the diagnosis of leukemia; with reference to this, we recall the case of a ten-month-old baby suffering from leukemia, whose blood contained a great number of these corpuscles, although the number of white corpuscles was hardly greater than 30,000."

Others before Hayem have successfully delved into hematologic problems, but I do not find it necessary to discuss to whom it is proper to attribute priority in conceiving one method of investigation or another for the description of a given technique. Every conception, every description will carry the personal imprint of the author. And what counts is the echo, the determining influence upon posterity. There will always be historians who discover the forerunners of men of genius.

While rendering respectful homage to other French pioneers of clinical hematology, Potain, Malassez, See, Vidal, Gubler, to cite only a few, we must realize that in this admirable phalanx, from the point of view of hematology, the work of Georges Hayem dominates from a great height, and his is the work that has prevailed. No clinician or investigator before him had, on the whole, as complete and comprehensive a view of the disorders of the blood.

Jolly said of Louis Malassez (and it could well be said of others) that he evoked in us the memory of men of a race almost extinct, of those artisans of the Middle Ages and the Renaissance, who, in the shadow of their workrooms, spent their lives perfecting and polishing some masterpiece with their own hands.

Georges Hayem, on the contrary, is of another mettle. Surrounded by pupils soon to become masters in their own right, he taught at the bedside of the sick or in the lecture room. He had this divine gift of the true professor to communicate, to transmit a brilliant synthesis of the past, together with his own discoveries and those of his contemporaries.

Is this not a sufficient reason for considering his hematology a unit filled with his "spiritual energy," kneaded with his own hands, as his original and personal masterpiece?

If many problems in hematology have changed in aspect, if we now hold different opinions from Hayem's on many questions, having at our disposal better means of investigation, it does not detract from the immensity of his work. He created the hematologic clinic, while hematology might have become and remained a pure morphologic science. As his own anatomopathologist he became the first hematologist.

He showed that, if the clinic without the laboratory is often an uncertainty, the laboratory without the clinic is always a danger.

Providence has put him in the right place by assigning to him a place in a clinic. Providence did better than could have done the secret wish of Hayem himself. Did he not wish to become the successor to Claude Bernard?

Who knows whether the brilliant synthesis of clinic and laboratory would have been evolved? For the strength of Hayem does not lie in abstract speculation (toward which he was driven by some secret longing in his soul), but in constantly confronting and correcting the soul's vision by clinical reality.

Rarely did a man have such clear, such precise vision of the highway that medicine would bestride. Hayem is a descendant of the prophets of Israel when he evokes in a stirring allegory his medicine at the crossroads. I could not forgive myself if I did not read it to you in part.

"Visualize the heads of our School, the eminent clinicians who are the honor of French medicine, as travellers who have walked for a long time and who, at the moment they believe they had come to the end of their tiring journey, about to take a well-deserved rest, suddenly see looming in their path a steep mountain. In their wake they have brought an army of pupils whom they have guided on this road. They are stopped as if by a barrier. These travellers, completely tired out, halt, but not all of them feel the same way about it. Up on the mountain, whose summit is invisible, a few men, taking a wholly different road, carry a flag and make signs of encouragement. Our travellers install themselves in the valley, and some say, 'We shall not go any further; the horizon we shall be able to see from here is sufficient for us. You who are young, climb on, follow these sturdy pioneers, and come back to tell us of the marvels you will encounter in their wake. We are sorry we have no more strength to complete this long and arduous journey.' Still others, luckily fewer in number, are prone to be skeptical and mocking. 'This road is not ours,' they say. 'This mountain is shrouded in clouds. You will be making a useless ascent. You will be like those excursionists who, upon reaching the summits of the Alps, are dazzled by the resplendent sun, and see nothing under their feet, the ground being hidden in opaque clouds.'

"At that time," he continues, "I was still among the young. My principal master was Vulpian, a standard-bearer, as remarkable a scientist as he was a clinician, an indefatigable worker who fell like a soldier on the battlefield. His hand pointed in the right direction. I could not hesitate.

"I thus continued on my way, but sought a path to my taste. Leaving my old masters in the valley, I did not wish to take new guides, and soon I was no longer disdained for being somewhat apart from the great road where the young were crowding, the newcomers, elbowing one another, stumbling over one another's heels.

"These joyous companions, gleefully climbing the mountain which had now become easily accessible, do not realize, having left the base, that the men of my generation needed a certain courage to undertake its ascent."

And he concludes: "The excursion is far from completed. To teach medicine in our day is to continue one's education. Isn't that what constitutes the incomparable charm of our profession?"

Hayem died at the age of 94. He was spared the sorrow of seeing his country in misery, his compatriots in distress. He was spared the humiliation of having the "*ius civitatis sine suffragio et honore*" applied to himself, seeing himself excluded from the French community because he was a Jew. He was spared the humiliation of being dismissed from the school, and from the Academy to whose renown he had devoted all his laborious life. He did not have to suffer the humiliating insults of the laws of discrimination. But what matters the decrees of an ephemeral government in the face of Hayem's genius?

I think Hayem would have found it sweet and satisfying to know that in the United States, where admiration for his work is so whole and sincere, there are united this evening men without prejudice, ardent defenders of liberty and human dignity, who are thinking of him with love and respect. He would have found it sweet and satisfying to know that he would have had among these men, on this hospitable soil, a second Fatherland.

# CLINICAL AND EXPERIMENTAL

## THE POLYCYTE\*

ERIC PONDER, M.D., D.Sc., MINEOLA, L. I.

IT IS generally recognized that the figures for Cooke's polynuclear count (Cooke, 1914) are related to the life history of the polymorphonuclear in the blood stream. In making the count 100 polymorphonuclear leucocytes are grouped according to the number of lobes in the nucleus of each; "if there is any band of nuclear tissue except a chromatin filament connecting different parts of a nucleus, the nucleus cannot, for the purposes of the polynuclear count, be said to be divided." This is called Cooke's criterion, and distinguishes the polynuclear count from the Arneeth count, from which it is derived. The count is then expressed (taking an average normal count as an example):

| I  | II | III | IV | V |
|----|----|-----|----|---|
| 12 | 25 | 44  | 15 | 4 |

Multiplying the number in class I by 1, the number in class II by 2, the number in class III by 3, and so on, and dividing by 100, one gets the mean for the count, in this case 2.74. The significance of the figures in the various classes lies in the fact that it has been shown that the cells of class I are young cells recently derived from the marrow, that increasing lobulation occurs during the lifetime of the polymorphonuclear leucocyte in the blood stream, cells of class I becoming cells of class II, cells of class II becoming cells of class III, and so on, and that the cells of the higher classes, IV and V, are preferentially removed by death (Cooke and Ponder, 1927; Ponder, 1926a, 1926b, 1927; Yeager and Haterius, 1929; Climenko, 1931; Climenko and Ponder, 1934). The normal state is represented diagrammatically in Fig. 1, the arrows indicating the delivery of young cells from the marrow, the removal of old cells by death, and development from class to class. The "shift to the left," characteristic of marrow stimulation, results from an increased production of young polymorphonuclear leucocytes from the marrow, as in acute infections, or from an increased death rate in the higher classes, as in chronic tuberculosis (Ponder, 1927). The successive classes are shown as unequal in length because a cell stays in class I a shorter time than in class II, in class II a shorter time than in class III, and so on.

In 1927 Cooke described a type of polymorphonuclear leucocyte for which it is difficult to find a place in the scheme. It is unusually large, and its nucleus is hypersegmented, sometimes having as many as from 6 to 12 lobes. Cooke gave it the name of macropolycyte, and subsequently classified macropolycytes into

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three types. Type I occurs very rarely in health,<sup>\*</sup> occasionally in infection, and in states where there is a marrow reaction, in experimental anemia in the rabbit, and in pernicious anemia. Types II and III resemble megakaryocytes and are found in pernicious anemia.

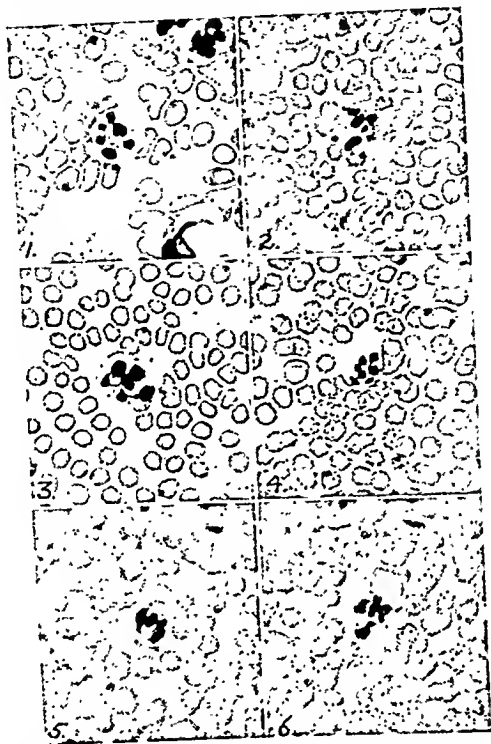


Plate I.—1, Polycyte from Case 1, corrosive fixation; 2, polycyte, Giemsa; 3, macropolycyte from Case 3, Giemsa; 4, polycyte from blood of guinea pig; 5, propolycyte (note the two very small lobes attached to the third large lobe), Giemsa; 6, propolycyte, Giemsa. (Magnification  $\times 344$ .)

#### POLYCYTES AND PROPOLYCYTES IN INFECTION

In acute infection, because of the concomitant marrow stimulation and the delivery of large numbers of young cells into the blood stream, the polynuclear count shows a large number of cells of class I and a left-handed deflection. As the cells of class I develop into cells of class II, and so on, the polynuclear count shifts to the right, and it is usually under such circumstances that I have observed the cells which I call polycytes and propolycytes.

1. *Polycyte*.—This is a polymorphonuclear leucocyte of the usual size, 10 to 14  $\mu$  in stained films,<sup>†</sup> but with a hypersegmented nucleus possessing from 6 to 12 lobes (Plate I). The lobes are often widely separated and joined by long chromatin filaments. One gets the impression that the lobes are unusually

<sup>\*</sup>No macropolycytes were observed in the blood of 90 persons who formed the basis of Cooke and Ponder's (1927) average normal polynuclear count. Cooke subsequently found one macropolycyte in one person, and reported to Kennedy that he had seen one in another healthy person. Kennedy and Mackay (1937) reported 9 in a series of 690 healthy males living in Iraq, and as many as 21 in 487 cases of leprosy, phlebotomus fever, and malaria.

<sup>†</sup>The size of the polymorphonuclear leucocyte in stained films depends very much on the method of fixation. Most of my material was stained by Giemsa after methyl alcohol fixation, but corrosive sublimate fixation and Heidenhain's iron alum is preferable for the study of polycytes and propolycytes. The cells are distinctly larger after corrosive fixation.

small, and some of the lobes may be very small indeed, looking like buds from larger lobes and connected to them by fine chromatin filaments.\*

One frequently sees in polyeytes an arrangement shown diagrammatically in Fig. 2 A, in which a fairly large lobe is joined by a chromatin filament to a very small lobe, which in turn is joined to another large lobe by another filament, apparently a continuation of the first. The separation of two pieces of nuclear material with a small piece left between them reminds one of the familiar Plateau's spherule, a very small drop of water which is formed between two larger drops as they separate (Edser, 1933) and which, when it occurs in a viscous fluid, is related to the phenomenon of "beading" in threads. It must be borne in mind that when two nuclear lobes separate, they do not move apart of themselves, but are drawn apart by some force, possibly the forces resulting from their movements through the fluid surrounding them. Given the proper relations of surface tension and viscosity necessary for beading, the appearance of the small lobe in the line of the filament joining two larger lobes is to be expected, and in this connection it may be observed that the appearance of polyeytes is often associated with an amoeboid appearance of the polymorphonuclear leucocytes, as if their cytoplasmic viscosity were reduced.

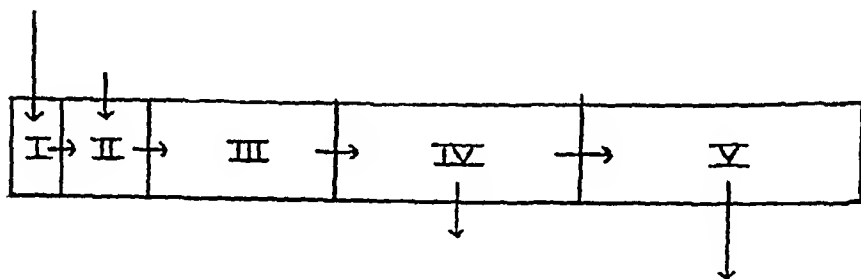


Fig. 1.—Diagrammatic representation of the life history of the polymorphonuclear leucocyte in the blood stream. For explanation, see text.

One also sees an arrangement, such as is shown in Fig. 2 B, in which one lobe is connected to two others by a branched filament. It is difficult to see how this can come about during the process of segmentation of the nucleus, and the possibility must be considered that the branching may be nothing more than an appearance, the "branches" being really two separate filaments lying close together for some distance from the lobe of origin. The filaments are so fine that two of them lying side by side may be impossible to resolve; nevertheless, there is often seen a barely recognizable speck of material at the junction of the branches, and the part of the filament which branches does not seem to be thicker than the filaments which proceed from it.

\*In Osgood and Ashworth's *Atlas of Hematology* there are two cells which resemble those described in this paper. No. 84 (neutrophile lobocyte) has 7 lobes and is from a case of myelogenous leucemia. Osgood says that some authors believe that such cells occur only in pernicious anemia and use this as a diagnostic criterion, but that they are also found in granulocytic leucemia, and occasionally in conditions in which the rate of leucocyte destruction is reduced. The cytoplasm is bluer than in the typical polymorphonuclear leucocytes, i.e., more like that of the "toxic" neutrophile as described by Osgood. No. 85 is from a case of pernicious anemia. Osgood describes this as the same as Cooke's macropolycyte, although the "macro" element is missing (the size is 15 by 12 $\mu$ , while that of a normal polymorphonuclear leucocyte at the upper limit of size, No. 83, is 15 by 15 $\mu$ ). Osgood suggests the term polylobocyte, and says that the size of such cells is often larger than that of the typical polymorphonuclear leucocyte. Since the distinguishing feature of Cooke's macropolycyte, type I, is its size, this illustration (No. 85 of the *Atlas*) is probably one of what I call a polycyte. Cooke's macropolycyte of type I would be similar in appearance, but larger (20 by 20 $\mu$ ).

In my experience polycytes are found in about 10 per cent of cases in which there has been a left-handed deflection of the polynuclear count followed by a commencing right-handed shift, i.e., at the stage of commencing recovery from a marrow stimulation. The following cases illustrate their appearance.

Case 1.—Traumatic amputation of arm and leg. Shock. Overwhelming toxemia from tissue injury. Patient died.

|   | AFTER ACCIDENT |              |        |
|---|----------------|--------------|--------|
|   | 1 HR.          | 5 HR.        | 18 HR. |
| White blood cells per cubic millimeter            | 13,725         | 31,900       | 5,600  |
| Polymorphonuclear leucocytes per cubic millimeter | 2,750          | 30,000       | 4,050  |
| Polynuclear mean                                  | 1.84           | 1.40         | 1.31   |
| Polycytes, per cent                               | 0              | Propolycytes | 2      |

Case 2.—Pulmonary tuberculosis. Thoracoplasty. Patient recovered.

|   | BEFORE OPERATION | AFTER OPERATION |        |
|---|------------------|-----------------|--------|
|   |                  | 12 HR.          | 24 HR. |
| White blood cells per cubic millimeter            | 19,978           | 21,500          | 21,125 |
| Polymorphonuclear leucocytes per cubic millimeter | 14,600           | 18,900          | 17,900 |
| Polynuclear mean                                  | 1.86             | 1.38            | 1.34   |
| Polycytes, per cent                               | 0                | 6               | 7      |

Case 3.—Perforated duodenal ulcer. Shock. Operation ten hours after perforation. Patient recovered.

|   | BEFORE OPERATION | AFTER OPERATION |                         |
|---|------------------|-----------------|-------------------------|
|   |                  | 19 HR.          | 6 DAYS                  |
| White blood cells per cubic millimeter            | 3,725            | 13,000          | 10,775                  |
| Polymorphonuclear leucocytes per cubic millimeter | 3,150            | 11,700          | 10,100                  |
| Polynuclear mean                                  | 1.04             | 1.32            | 1.54                    |
| Polycytes, per cent                               | 0                | 4               | , and 2 macro-polycytes |

2. *Propolycyte*.—This is a polymorph of the usual size, characterized by a nucleus with several lobes, but of a quite unusual degree of complexity. These cells have, indeed, all the characteristics of polycytes, except that their nuclei are not sufficiently hypersegmented. The arrangement of the nuclear lobes, however, is like that seen in the polycyte; the lobes are often small and joined by conspicuously long chromatin filaments, and with respect to one another they lie in patterns not seen in the typical polymorphonuclear leucocyte. Some examples are shown in Plate I.

In health typical propolycytes are rarely seen, but between them and the typical polymorph there exists a whole series of forms, usually referred to as "complex forms" (Ponder and Flinn, 1926). Certain apparently normal persons show few polymorphonuclear leucocytes with three, four, and more nuclear lobes, and yet have a great many complex forms which have to be grouped in classes I and II if Cooke's criterion is adhered to strictly. The same condition is met with in certain animals.\*

\*In the mouse the polymorphonuclear leucocytes are complex forms, although by Cooke's criterion, they fall into classes I and II. In the guinea pig, cells with as many as 10 lobes occur, and the polynuclear mean is about 5.0. In some of the marsupials, e.g., the wombat, the mean is as high as 5.0 if all cells with more than 6 lobes are considered as cells of class VI. It would be still higher if proper weight were given to cells of the higher classes.

In conditions of abnormal marrow activity propolycytes occur under the same conditions as do polycytes, and are almost certainly a stage in the development of the latter. Thus in Case 1, above, propolycytes preceded the appearance of polycytes, just as in Case 3 polycytes preceded the appearance of macropolycytes.

#### A CASE OF THE DEVELOPMENT OF POLYCYTES IN VITRO

The phenomenon of polycyte development in vitro deserves a detailed description because the possibility of its occurring has not hitherto been suspected.

The blood was that of a man, aged 53 years, who later had a partial gastrectomy performed for ulcer. In heparinized venous blood withdrawn at 1:20 P.M. on Feb. 26, 1941 (a week before operation), he showed a total white blood cell count of 13,375, with 96 per cent polymorphonuclear leucocytes, and a polynuclear count of

| I  | II | III | IV | V |
|----|----|-----|----|---|
| 10 | 38 | 44  | 6  | 2 |

with a mean of 2.52. For a reason entirely apart from the subject of this paper, films were made on both slides and cover slips at 2:30 P.M., 4:00 P.M., and 6:15 P.M., during which time the small bottle containing the heparinized blood stood on the laboratory bench at a temperature which varied between 22.3° C. and 22.7° C. Subsequent counts on the stained films showed polycytes in the following numbers:

TABLE I

| TIME<br>(t) | NO. ON SLIDES                                  | NO. ON COVER<br>SLIPS | MEAN NO.<br>IN 400 |
|-------------|--|-----------------------|--------------------|
| 1:15 P.M.   | 1 in 400<br>2 in 400                           | -                     | 1.50               |
| 2:30 P.M.   | 3 in 400<br>2 in 400<br>5 in 400<br>3 in 400   | -<br>-<br>-<br>-      | 3.25               |
| 4:00 P.M.   | 1 in 400<br>6 in 400                           | 1 in 400<br>6 in 400  | 3.50               |
| 6:15 P.M.   | 19 in 400<br>9 in 400<br>9 in 400<br>11 in 400 | -<br>-<br>-<br>-      | 12.00              |

The most convenient way of deciding whether or not the numbers found at the various times can be accounted for by variations in a random sample is to use a method employed in testing defective production of materials (American Society for Testing Materials: *Manual on Presentation of Data*, Section 17). The mean number of polycytes (5.57) and its standard deviation ( $\pm 2.34$ ) are calculated for the 5,600 cells counted, and the distribution of the polycytes, with lines marked for the mean number and for  $2\sigma$ ,  $3\sigma$ , etc., are plotted in Fig. 2. The odds for points lying between the  $2\sigma$  lines are 20 to 1 or better that they are the result of random sampling of one uniform population of 5,600 cells, the odds corresponding to  $3\sigma$  about 300 to 1, to  $4\sigma$ , 10,000 to 1, and so on. The odds that 19 polycytes would be found in a sample of 400 cells taken at random from the blood under consideration are those corresponding to  $5.7\sigma$ , or about 1 in a hundred million.

Looking at the figure, the trend toward larger numbers of polycytes at the later times is apparent, and the solid central sloping line is that of the equation  $N = 1.82t + 1.5$ , drawn by the method of least squares. On either side of it are two parallel lines for twice the standard deviation ( $\pm 1.46$ ) of the points about this line. All the points lie between these two lines, except the very high point ( $N = 19$ ) at 6:15 P.M. and the two low points ( $N = 1$ ) at 2:30 P.M. Even in the case of these three points the deviation is in the right direction; hence the trend is a real one.

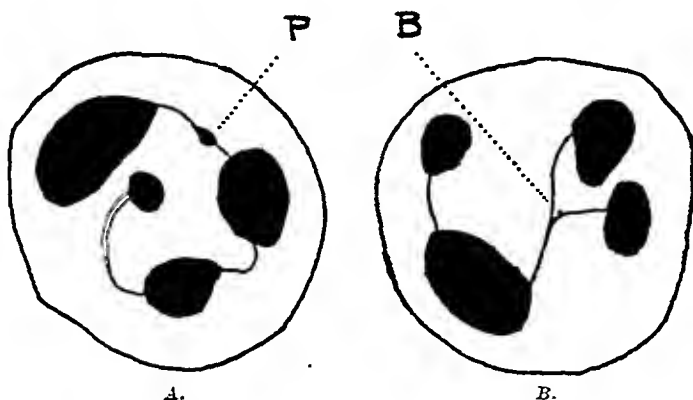


Fig. 2.—Diagrammatic representation of two polymorphs, one showing a small lobe (*P*) between two large lobes, and the other showing a branched filament *B*. For discussion, see text.

This is the only case in which I have followed an *in vitro* development of polycytes in sufficient detail to be certain of it, but on several occasions I have observed (without statistical proof) an *in vitro* increase in the number of propolycytes. The process is all the more remarkable in view of the difficulty encountered in showing any development from class to class in the blood stream of the normal animal (Climenko and Ponder, 1934). Finally, reference should be made to an observation (Ponder and MacLeod, 1938) in which, on one occasion, the blood stream of a rabbit injected with nucleic acid became flooded with macropolycytes within two hours.

#### DISCUSSION

It is apparent that the propolycyte, the polycyte, and the macropolycyte have a place in any scheme which describes the maturation of the polymorphonuclear leucocyte, and also that the present scheme (Fig. 1) requires some essential modification. I worked out this life history in 1926 by observing the effect of thyroid injections on the polynuclear count in rabbits; the progressive development from class to class was demonstrated, and the whole development from class I to class V was found to take from two to three weeks (Ponder, 1926). This result was confirmed, in general, by Ponder and Flint (1927, deflection with various drugs and extracts, including nucleic acid); Kennedy and Grover (1927, deflection with x-rays); Kennedy and Thompson (1927, deflection by ultraviolet light); Charipper (1928, deflection by thyroid extract in *Necturus*); Danzer (1930, deflection by tissue extracts); and Climenko (1930, deflection by ergosterol and ultraviolet light). In all these cases it was found that the polynuclear count returned to its original steady state after from fourteen to twenty-one days. All these experiments were on rabbits.

MacLeod and I have recently found that the production of sterile peritoneal exudates in rabbits results in a deflection of the count, and that a return to the original steady state takes about seven days, while the return takes only about four days when the deflection is the result of the injection of nucleic acid (1 mg. per kilogram). We explained this on the basis that the polynuclear counts in the rabbits we used were more left handed than those of the animals in the experiments conducted from 1926 to 1930, but the fact is that the time required for a return to the steady state is more variable, and often shorter, than the early experiments indicated.

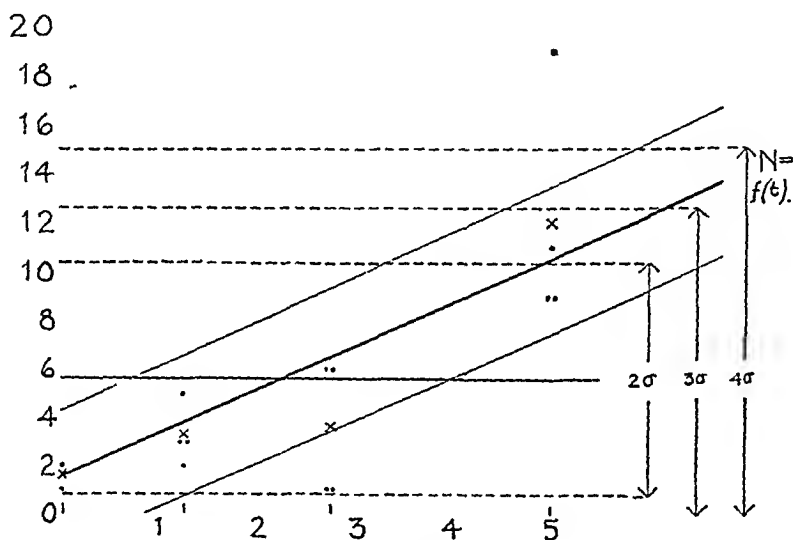


Fig. 3.—Number of polycytes plotted against time. For explanation, see text.

The simplest way of reconciling these various observations is to suppose that the rate of maturation of the polymorphonuclear leucocyte, as measured by the lobulation of its nucleus, may vary. Cooke (1934) did not consider this possibility specifically in his two suggested explanations for the presence of macropolycytes, the first being an escape from the destructive processes which usually dispose of the cell at the stage of class IV or V, and the second being an altered development due to exposure to an abnormal environment during early development. This second suggestion might cover an increased rate of lobulation. I have considered the converse situation, a decreased rate of maturation, in connection with the steady state in chronic infections, and have dismissed it as applying to that state (Ponder, 1927). An increased rate of passage from class to class, however, would obviously account for the appearance of polycytes and their predecessors, propolycytes. This is what appears to occur in about 10 per cent of the type of case referred to above, and in some cases segmentation is so rapid as to produce polycytes within a few hours.

It should be borne in mind, however, that the macropolycyte of Cooke is unusual because of size as well as hypersegmentation (which may or may not be present in macropolycytes of types II and III; Cooke, 1934). This element of size, without the hypersegmentation, appears in the polymorphonuclear

leucocytes of rabbits with sterile peritoneal exudates and after the injection of nucleic acid (Ponder and MacLeod, 1938), whereas it is more usual in cases of infection to find the appearance of the element of hypersegmentation without that of size. Two factors thus seem to influence the development of the polymorphonuclear leucocyte in the blood stream, and these may operate while the cell is in its early stages, perhaps as early as the stage of the hemocytoblast: the factor for segmentation, and the factor for size. The former is involved in the production of propolycytes and polycytes; the latter, in the production of the kind of polymorphonuclear leucocyte found after peritoneal exudates and the injection of nucleic acid in the rabbit. Both, acting together, are involved in the production of macropolycytes of type I.

The idea of a change in the rate of maturation of the polymorphonuclear leucocyte may be applied to the regional and seasonal variations in the polynuclear count described by MacLeod (1935, 1938), and to the unusual degree of segmentation found in the polymorphonuclear leucocytes of some animals, such as the guinea pig (Curphey and Ponder, 1941) and the wombat (Ponder, Yeager, and Charipper, 1928). The extraordinarily rapid return of the polynuclear count in the guinea pig to its initial steady state after a deflection produced by nucleic acid is strong enough evidence that the life history of the polymorphonuclear leucocyte in this species is different from that of the rabbit and of man.

#### SUMMARY

1. This paper discusses the relation of two types of polymorphonuclear leucocytes, the polycyte and the propolycyte, to the typical polymorphonuclear leucocyte on the one hand and to the macropolycyte on the other. Polycytes and their precursors, propolycytes, are characterized by hypersegmented or complex nuclei, but are of the usual size (about  $15\ \mu$  diameter).

2. Polycytes and propolycytes are probably the result of an increase in the rate of maturation of the polymorphonuclear leucocyte, and a case of their development in vitro is described.

3. These cells make their appearance in about 10 per cent of cases with acute or chronic infection, usually when the polynuclear count is returning, or about to return, to its normal steady state. In general, they are of favorable prognostic significance, and their appearance in states of infection suggest that under such circumstances the rate of segmentation of the polymorphonuclear leucocyte may be markedly increased as a result of factors acting on the cells at some earlier stage of their development.

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## RECOVERY FROM EXPERIMENTAL POLYCYTHEMIA\*

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POLYCYTHEMIA is generally conceded<sup>1</sup> to be due to increased bone marrow activity. However, in 1929 (Goldbloom and Gottlieb<sup>2</sup> in their studies on ieterus neonatorum reported increased serum bilirubin and raised icterus indices in guinea pigs returned to normal atmospheres after being made polycythemic by exposure to low atmospheric pressures. This, apparently, confirmed observations on hemolysis of blood in plethora due to transfusion<sup>3</sup> and seemed to indicate a regulatory, hemolytic control of the level of circulating erythrocytes. It would suggest that a derangement of this mechanism is a requirement for chronic polycythemia vera. An attempt was made to study the site of this hemolysis, but the report indicates failure to verify the fundamental observation of Goldbloom and Gottlieb.

### METHODS

Twenty-one male guinea pigs, weighing 550 to 880 Gm., were used. Erythrocyte counts, hemoglobin in grams (by Evelyn photoelectric colorimeter<sup>4</sup>), and reticulocyte counts (brilliant cresyl blue on dry smear) were made at the outset, blood being obtained by pricking a small ear vein. The animals were then placed in the low pressure chamber, where the pressure was reduced gradually over the first week to 350 mm. Hg below atmospheric pressure, i.e., 410 mm. Hg, corresponding to an altitude of 16,000 feet<sup>5</sup> and maintained at this level for the ensuing two weeks. The apparatus was that described previously.<sup>3</sup> The animals were removed daily for perhaps fifteen minutes for cleaning of the chamber and introduction of fresh food, and weekly for about an hour for the above blood determinations. At the end of this period the animals were returned to atmospheric pressure, hematologic determinations were made, one animal of each group was killed as control, and the remainder were sacrificed in series during the next week, or during longer time in a few cases. Blood studies were made every forty-eight hours on the whole group, or just before death when one animal was killed on an alternate day. About 5 c.c. of blood for bilirubin determination were withdrawn by cardiac puncture, after which the animal was

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killed and the spleen, liver, and femoral bone marrow were removed. The spleen was weighed fresh, and sections of spleen and liver were fixed and stained for iron. Sections of bone marrow were stained by Giemsa's method. Bilirubin readings were made on the Evelyn photoelectric colorimeter by the method given by Malloy and Evelyn.<sup>6</sup>

Since Goldbloom and Gottlieb removed 0.5 c.c. of blood every forty-eight hours, it was thought that the reticulocytosis dependent on chronic blood loss might have contributed to hemolysis in their experiments. Accordingly, 5 guinea pigs were so treated at low atmospheric pressure. It was also considered possible that their pigs had a larval anemia due to diet, so that 4 guinea pigs were placed on the diet described by Jacobson.<sup>7</sup> These animals responded to low pressure as did normal controls, previously observed.<sup>8</sup>

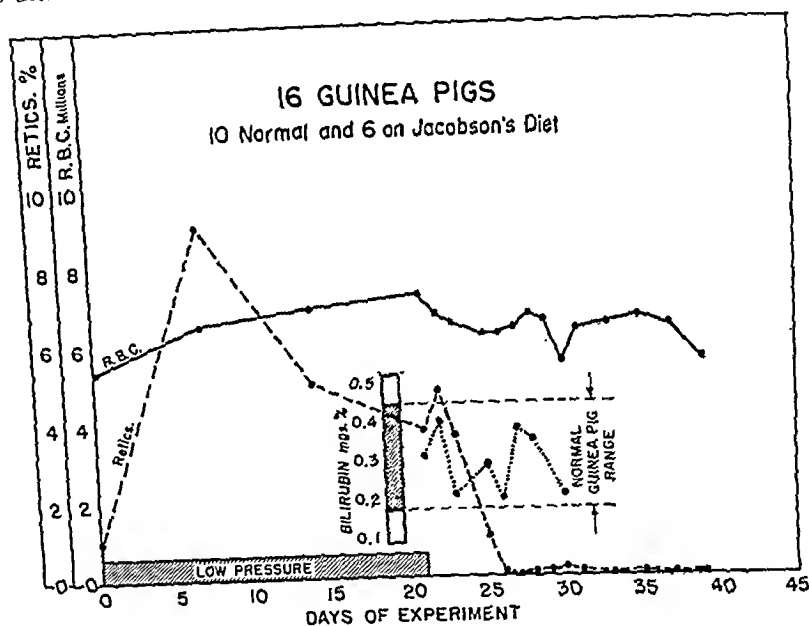


Fig. 1.—Averaged responses of 16 guinea pigs. Those on the diet responded as did normal guinea pigs. Hemoglobin values were parallel to red blood cell counts and are not shown.

#### RESULTS AND DISCUSSION

It is seen that in all types of experiment the red blood cell count and hemoglobin were sharply elevated in response to low pressure. A reticulocyte peak occurred somewhat before the peak of this response. Upon return to normal pressure red blood cell count and hemoglobin fell gradually to normal, preceded by a decrease of circulating reticulocytes. These results are shown in Figs. 1 and 2. The experiment was terminated too early to observe the anemia described at about one month in guinea pigs by Gordon and Kleinberg,<sup>9</sup> and in rats by Tyler and Baldwin.<sup>10</sup> The bone marrow from marked hyperplasia became more quiescent, a few fat vacuoles becoming evident in section, and circulating reticulocytes practically disappeared, except after chronic blood loss, while erythrocytes and hemoglobin levels were still elevated. At no time was any increase in serum bilirubin demonstrable. The spleen and liver showed no histo-

logic increased iron content during recovery. No quantitative urobilin excretion studies were undertaken, since the more easily obtained and negative evidence was considered to preclude the necessity for this. No explanation is offered as to why none of the animals showed the rise in serum bilirubin which Goldbloom and Gottlieb observed in similar experiments, and which they interpreted as explaining *icterus neonatorum*.

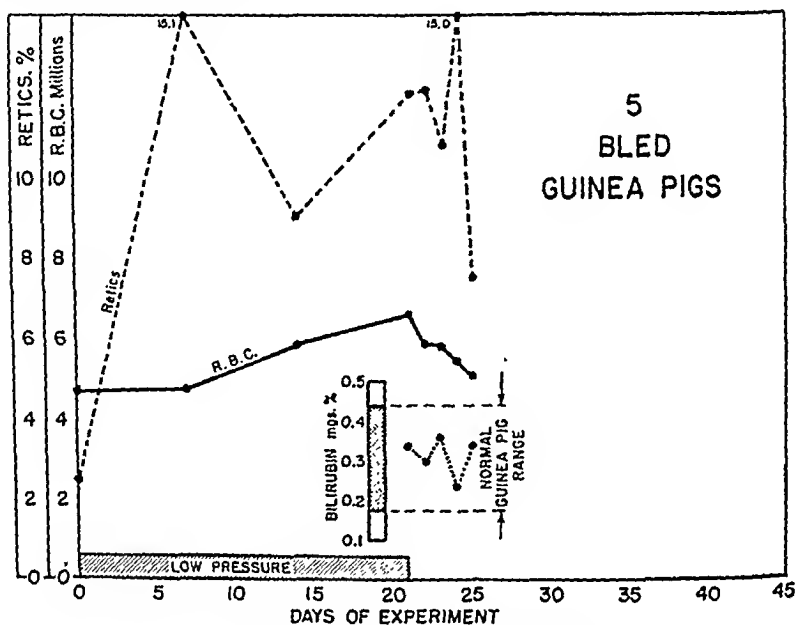


Fig. 2.—Averaged responses of 5 guinea pigs bled every forty-eight hours. Hemoglobin values were parallel to red blood cell counts.

This "physiologic" *icterus* has presented an interesting problem, beyond the scope of the present discussion. Goldbloom and Gottlieb<sup>11</sup> considered it due to increased fragility of infants' blood, dependent on high per cent of reticulocytes, which cells they thought particularly fragile. However, increased fragility of the blood of the newborn or of reticulocytes was verified neither by Whitby,<sup>12</sup> using precise methods, nor by Mitchell.<sup>13</sup> The latter found bilirubin raised at birth and abundant iron in the placenta, but none in the reticulo-endothelial system, this iron appearing later. He concluded that the process producing bilirubinemia must start before birth. A possible explanation of this process is afforded by Barcroft,<sup>14</sup> who showed that fetal hemoglobin has a different dissociation curve, with a slightly greater affinity for oxygen than that found after birth. This observation is corroborated by chemical analysis and by spectroscopy.<sup>14</sup> A process might occur whereby fetal hemoglobin is replaced by an adult type, excess iron being eventually stored in liver and spleen. Waugh, Merchant, and Maughan<sup>15</sup> have recently reviewed this subject and have shown that in the newborn, in whom there is normally accelerated blood destruction, there is no correlation between the rate of fall of blood levels and the degree of bilirubinemia. They consider their evidence consistent with a hepatic origin of the *icterus*. At any rate, *icterus neonatorum* and normally rapid blood destruction in the newborn are not examples of a reaction which in adults might

cause bilirubinemia after previous exposure to low oxygen tension. Acceleration of blood destruction or rise in serum bilirubin has not been demonstrated in adult mammals changed from low to normal oxygen tension.

### CONCLUSIONS

In the guinea pig recovery from polycythemia induced by low atmospheric pressure appears dependent on subsidence of increased bone marrow activity rather than on excess peripheral destruction of erythrocytes. No rise of serum bilirubin was found, even in animals in which blood regeneration had been altered by bleeding or by special diet while at low oxygen tension.

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## STUDIES ON VASODILATATION TESTS IN PERIPHERAL VASCULAR DISEASE\*

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THE fact that functional states may simulate organic involvement of the peripheral arterial tree has long been recognized. This has led to the devising of various tests for differentiating organic narrowing or occlusion of peripheral vessels (a permanent, nonreversible change) from narrowing due to vessel spasm (a transient, reversible change).

This differentiation, moreover, is important not merely for diagnostic reasons, but for its prognostic and therapeutic implications as well. Organic involvement, as in arteriosclerosis obliterans or thromboangiitis obliterans, implies a long-drawn, laborious effort to establish and to extend collateral circulation. The prognosis, even in cases of moderate involvement, is not too bright. In functional cases, however, the problem of releasing vessel spasm can be solved in most instances by physical or chemical means; in any case, the possibility of the involvement progressing to the death of tissue is less likely.

In addition there is the large group of cases where spasm and organic encroachment on vessel lumen coexist to varying degrees, and here the problem has been to establish the respective roles of the two causative mechanisms. The three tests here reported on are (1) the thermal reflex vasodilatation test; (2) the peripheral nerve block; and (3) the sodium nitrite test.

The thermal reflex vasodilatation test is based on the physiologic fact that when excessive heat is applied to a part of the body, the body attempts, through a compensatory generalized cutaneous vasodilatation, to dissipate this heat. The mechanism appears to be a reflex release of sympathetic vasoconstrictor influence. This vasodilatation, manifested peripherally by elevation in skin temperature of the extremities, is measured with a thermocouple.

Complete vasodilatation, under the standard conditions of the test,<sup>1</sup> is taken to indicate normal patency of peripheral arteries in the extremity tested, with no appreciable organic encroachment on the lumen of these vessels. However, we have found that failure of an extremity to show maximal vasodilatation is not invariably due to organic obliterative involvement; it may be an expression of a degree of vasoconstrictor tone (spasm) so high that the thermal stimulus is not sufficient to undo it. Every such failure in a proved nonorganic case of vessel spasm is, of course, a mark against the reliability of the thermal test.

Peripheral nerve block is a direct attack on the sympathetic innervation of the foot or hand, aiming at local vasodilatation. In the lower extremity the posterior tibial nerve at the ankle and the common peroneal nerve at the neck

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of the fibula are blocked with novocain. In the upper extremity the median nerve at the wrist and the ulnar nerve at the elbow are similarly blocked. The resulting abolition of sympathetic control manifests itself in vasodilatation distal to the level of block, and the rise in skin temperature is again measured as in the thermal test.

The sodium nitrite test<sup>2</sup> exploits the well-known relaxing effect of nitrites on the smooth muscle of peripheral blood vessels generally. The dose of sodium nitrite given intravenously is kept small enough to obviate any secondary vasoconstrictor manifestations: 1 c.c. of 4 per cent sodium nitrite for the adult of average size. The major peripheral effect in this test is not an increase in blood flow, as in the thermal test and in peripheral nerve block, but an increase in pulsating volume of the limb, and a pooling of blood in the venocapillary bed.<sup>2</sup> There is thus no rise in skin temperature suitable for thermocouple determinations. The effect is tested with the oscillogram, which records the increased amplitude of pulsation when this occurs. The increase in oscillations is taken as a measure of the degree of spasm present before the test, and the failure of oscillations to increase indicates advanced organic involvement, with little or no coexisting vessel spasm.

The object of this study was to investigate the capacity of each of these three procedures to abolish vessel spasm and to induce in the limbs tested the maximal vasodilatation of which they were capable. We wished to establish the relative dependability of these tests as indicators of vessel spasm, and hence to derive definite impressions of the place of these tests in the study of peripheral vascular disease.

Our clinical material included 75 patients with a variety of peripheral vascular disorders. Each of these patients, in addition to a clinical estimate of his peripheral vascular status, was subjected to at least two of the foregoing tests one or more times. The clinical evaluation was arrived at on the basis of a complete history and physical examination, and a comprehensive examination of the extremities, including observations on rubor, pallor, temperature, peripheral pulses, nutritional changes, oscillogram determinations, claudication studies, and x-rays. The cases were, with few exceptions, reviewed at staff conferences, so that the clinical classification represented the consensus of the clinical staff.

In correlating with these clinical findings the results of the thermal, nerve block, and nitrite tests, we have found etiologic distinctions alone unsatisfactory. We have grouped the patients on the basis of the degree of vascular impairment,<sup>3</sup> since the value of these tests lies not in differentiating between the various peripheral vascular ailments, but in indicating the latent vascular reserve of the patient.

Of the 75 patients included in this study, 15 were in the IIA group, i.e., they had organic involvement with minimal symptoms, with varying degrees of associated vessel spasm; 32 were in the IIB group, i.e., they had more advanced organic involvement with moderate symptoms, but were short of open lesions or gangrene; 28 were in the functional group (class IV),<sup>3</sup> with normal vascular reserve and no demonstrable organic disease. In the latter group the symptoms were entirely due to vessel spasm.

Table I represents in summary the relative dependability of the three tests. Of 35 thermal tests done on patients in the functional group, only 11 revealed the full degree of the vascular reserve in the limbs tested. Twenty-four of the 35 thermal tests (69 per cent) failed to induce the maximal vasodilatation which was attained in response to other tests. Thus, in one patient after another whose symptoms, from a clinical viewpoint, seemed due solely to vasospasm, reliance on the thermal test alone would have led to the wrong conclusion that organic vascular disease was present.

TABLE I

| GROUP      | THERMAL      |                               |            | NITRITE      |                               |            | BLOCK        |                               |            |
|------------|--------------|-------------------------------|------------|--------------|-------------------------------|------------|--------------|-------------------------------|------------|
|            | NO. OF TESTS | NO. WITH MAX. VASO-DILATATION | % SUC-CESS | NO. OF TESTS | NO. WITH MAX. VASO-DILATATION | % SUC-CESS | NO. OF TESTS | NO. WITH MAX. VASO-DILATATION | % SUC-CESS |
| Functional | 35           | 11                            | 31         | 17           | 12                            | 70         | 24           | 24                            | 100        |
| IIA        | 23           | 6                             | 26         | 6            | 3                             | 50         | 13           | 13                            | 100        |
| IIB        | 37           | 20                            | 54         | 23           | 10                            | 43         | 29           | 28                            | 96         |

It may be seen at a glance that the best showing of the thermal test was in the group IIB cases. In this group patients with advanced organic obstruction of vessels showed a minimal capacity for vasodilatation to begin with, and hence the inconstancy of the thermal stimulus to release spasm is obscured. In the IIA and functional groups, however, this weakness of the thermal test becomes conspicuous. For patients in these groups latent capacity for vasodilatation is high, and inadequate response to the thermal test repeatedly was followed by complete, or almost complete, vasodilatation after peripheral nerve block.

The reliability of the nitrite test paralleled roughly that of the thermal test in the IIB group, but was considerably ahead of the thermal test in the functional group. The greater incidence of failure in the subjects with advanced organic involvement may be due to the fact that the nitrite test measures increase in pulsating volume; and sclerotic, rigid, inelastic vessels are less capable of enhanced pulsation than the structurally normal arteries of the functional cases. In cases with no organic disease, where spasm alone was the cause of symptoms, the nitrite test was more dependable, and gave accurate indication of the actual vascular reserve in 70 per cent of cases.

Peripheral nerve block, properly performed and confirmed by the development of anesthesia or paralysis along the distribution of the nerves, was, in our experience, by far the most accurate and dependable test for vasospasm. With the exception of only one case, we found no instance where vasodilatation in response to any other measure exceeded the vasodilatation following nerve block.

Table II represents the results in a number of patients, each of whom was subjected to thermal test and nerve block done in a parallel fashion, i.e., under similar conditions and within several days of each other, to obviate the possibility of a change in vascular status between tests. Again, whereas the thermal test was accurate in only 30 per cent of the functional cases, and in 46 per cent of the IIB cases, nerve block was almost unfailingly effective in inducing the maximal vasodilatation possible in the limb tested.

TABLE II

| GROUP      | NO. OF<br>PARALLEL<br>STUDIES | THERMAL                              |              | BLOCK                                |              |
|------------|-------------------------------|--------------------------------------|--------------|--------------------------------------|--------------|
|            |                               | NO. WITH<br>MAX. VASO-<br>DILATATION | %<br>SUCCESS | NO. WITH<br>MAX. VASO-<br>DILATATION | %<br>SUCCESS |
| Functional | 23                            | 7                                    | 30           | 23                                   | 100          |
| IIA        | 13                            | 3                                    | 23           | 13                                   | 100          |
| IIB        | 28                            | 13                                   | 46           | 27                                   | 96           |

In Table III are summarized parallel studies of nitrite test and nerve block. The results once again confirm the consistent accuracy of peripheral nerve block.

TABLE III

| GROUP       | NO. OF<br>PARALLEL<br>STUDIES | NITRITE                              |              | BLOCK                                |              |
|-------------|-------------------------------|--------------------------------------|--------------|--------------------------------------|--------------|
|             |                               | NO. WITH<br>MAX. VASO-<br>DILATATION | %<br>SUCCESS | NO. WITH<br>MAX. VASO-<br>DILATATION | %<br>SUCCESS |
| Functional  | 10                            | 6                                    | 60           | 10                                   | 100          |
| IIA and IIB | 17                            | 5                                    | 29           | 16                                   | 95           |

Table IV shows the results of thermal and nitrite test studies done in parallel manner. While in the patients with organic damage the nitrite test falls below the thermal test in dependability, it is approximately twice as accurate as the thermal test in functional cases.

TABLE IV

| GROUP       | NO. OF<br>PARALLEL<br>STUDIES | THERMAL                              |              | NITRITE                              |              |
|-------------|-------------------------------|--------------------------------------|--------------|--------------------------------------|--------------|
|             |                               | NO. WITH<br>MAX. VASO-<br>DILATATION | %<br>SUCCESS | NO. WITH<br>MAX. VASO-<br>DILATATION | %<br>SUCCESS |
| Functional  | 16                            | 7                                    | 43           | 13                                   | 81           |
| IIA and IIB | 29                            | 16                                   | 55           | 12                                   | 41           |

## SUMMARY

In the study of the patient who presents himself with symptoms referable to the peripheral arterial tree, all three tests discussed have a place. The place for each is indicated in the results we have summarized in the accompanying tables.

Sodium nitrite injected intravenously and the hot water bath are both useful as preliminary tests for vasospasm. Of the two, the thermal test is better suited to patients with organic vascular involvement when we wish to discover the degree of associated vessel spasm. In patients who give the clinical impression of nonorganic vasospastic involvement the nitrite test is considerably more dependable. It is also more feasible for patients prone to syncope in a hot bath.

In our clinic a patient whose vasodilatation in response to either of these tests reaches normal values is then regarded as possessing normal vascular reserve. Those who respond to either of these tests with subnormal vasodilatation are then tested with peripheral nerve block. The advantage of this scheme is that in a considerable proportion of patients a simple, nonmanipula-

tive procedure serves adequately to reveal the intensity of spasm and the vascular reserve of the limb in question. Nerve block is reserved as the test of last appeal for those patients in whom either the thermal stimulus or the sodium nitrite failed to induce fully normal vasodilatation.

We wish to acknowledge the participation of Dr. Abraham Kahane and Dr. Abraham Katz in the performance of the tests described.

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### PRIMARY AMYLOIDOSIS OF THE LUNGS\*

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AMYLOID disease, as is well known, usually follows certain chronic diseases, such as tuberculosis, and is characterized by massive and easily recognized lesions of the spleen, liver, kidneys, and adrenal glands. This is the secondary and typical form. It is less well known that there are rare, atypical, so-called primary forms of this disease which are seldom recognized clinically. The criteria for this atypical group were pointed out by Labarsch<sup>1</sup> and are (1) Almost complete absence of amyloid in organs most involved in typical amyloidosis, such as spleen, liver, and kidneys. (2) Presence of amyloid in organs and parts not usually involved, such as heart, lungs, and skin. (3) The occasional occurrence of tumor-like nodules of amyloid. (4) The frequent failure of the deposits to react to the specific stains or tests for amyloid. (5) The absence of a preceding or concomitant disease to which the presence of amyloid may be ascribed. The case report herewith cited is an excellent example of the primary or atypical form and illustrates the lack of clinical awareness of this condition.

#### REPORT OF CASE

The patient was a woman, aged 51 years, who had three outstanding complaints: extreme fatigue on the least exertion, dyspnea even at rest, and upper abdominal distress.

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Her family history was unimportant. She had had no chronic illness of any sort or any previous serious acute illness. She had no peculiarities of appetite, and no drug habits. She at no time had any symptoms referable to her tongue. In 1925 she had a tonsillectomy. In 1927 she received radium treatment for uterine fibroid. Menstruation continued until 1935.

Three years ago she noticed some thickening of the eyelids, and subsequently a similar condition involving the external auditory canal. Next there was thickening of the skin in the groin, which extended down to the vulva and rectum. In September, 1939, she was treated by a physician for arthritis involving the right knee and received four injections of lipoid iodine solution into the joint. Since April, 1940, she suffered from increasing dyspnea, and until near her death she was quite dyspneic when at rest in bed. She was hospitalized in September, 1940, for a study of the skin lesions. In the hospital these lesions were observed as yellowish brown elevations or infiltrations involving both upper and lower eyelids on both sides; as yellowish brown thickenings narrowing the lumina of the auditory canals almost to complete closure; and in the groins, not as nodular or glandular enlargements, but as wide areas of infiltration extending around to the rectum. The dermatologist made a diagnosis of xanthoma, and a biopsy was done on the eyelid and groin lesions. The pathologic report on these tissues was not definite. It was stated "that the tissue was probably originally xanthomatous but did not show that condition clearly now." The hyaline or homogeneous appearance of the subepidermal tissue in both eyelid and groin tissues was noted, but the real condition was not recognized. In October, 1939, a biopsy was done on the occluding lesion of the ear canal. The pathologist who examined the specimen also consulted a dermatologic pathologist on the diagnosis, and the joint opinion was colloid degeneration of the skin.

In the interval between April, 1940, and January, 1941, the patient consulted many physicians without diagnostic satisfaction, though she was assured she had no heart trouble. She became steadily worse, and a nonproductive cough was added to her increasing dyspnea. She was again hospitalized on Jan. 14, 1941, when the following data were recorded:

The patient was a large woman, about 6 feet tall, weighing over 200 pounds. She exhibited no cyanosis or jaundice, but was patently dyspneic at rest. She presented a pale and waxy appearance, with some swelling of the feet. Her mouth was clean and her teeth were good. Her tongue was moist and subjectively and objectively negative. Her heart sounds were weak but regular, and no murmur was detected. The systolic pressure was 134 mm. but the diastolic pressure could not be satisfactorily read. The electrocardiogram showed coronary T-waves in Leads I and II. No rales or other abnormalities were detected over the lungs. The roentgenogram showed some enlargement of the cardiovascular silhouette, and the lung fields were indefinitely hazy. There was some tenderness over the gall bladder and duodenum, but the liver and spleen were not palpable and there was no abdominal mass. The urine on all occasions showed considerable albumin and hyaline and granular casts. Urinary concentration was good. The hemoglobin was 12.8 Gm., the red blood cell count was 4,160,000, and the white blood cell count was 6,900. The differential count was normal. Blood sugar was 105 mg., blood urea 14 mg., blood creatinine 1.2 mg., and cholesterol 187 mg. The Wassermann and Kahn tests were negative.

After five days in the hospital the patient died suddenly and quietly without fever, rise of pulse rate, or signs of heart failure.

*Autopsy.*—Body that of an obese, white female of about 50 years. Nodular infiltrations of eyelids and groins, and slight edema of lower extremities, were noted.

The pericardium was normal in appearance. The heart was generally enlarged, weighing 510 Gm. There was moderate hypertrophy of both ventricles, the left ventricle measuring 18 mm. and the right 8 mm. in thickness. Otherwise auricles and ventricles appeared normal. There was no scarring grossly apparent, and there was no stiffening or fixation of the auricular walls. The orifices and valves were normal. The coronary arteries showed some atherosclerosis but no occlusion. The ascending portion of the thoracic aorta showed minimal atheromatous change; the abdominal aorta exhibited a moderate amount.

The lungs, in situ and on removal from the body, presented an expanded appearance without any tendency to the usual post-mortem collapse. The right lung weighed 860 Gm. and the left lung 810 Gm. They were dry, pinkish in color, and showed no focal changes, no areas of pneumonic consolidation or tuberculosis, and no edema. The cut surface had a fine honeycombed appearance, the empty alveoli remaining expanded. In appearance and consistency the lungs resembled a fine rubber sponge. Pleural cavities were normal. Tracheal and bronchial passages were free.

The spleen (150 Gm.) and the liver (1,810 Gm.) were without noteworthy gross changes. The gall bladder and ducts were normal. The pancreas was unchanged. The adrenal glands were apparently normal. The kidneys were embedded in considerable fat, each weighing 100 Gm. and showing some surface-pitting. The capsules stripped easily, revealing slight granularity. On section the cortex-medulla ratio was about normal. No abnormal findings were noted in the ureters or bladder. The uterus presented on the posterior surface a subserous fibroid, 1.8 cm. in diameter; uterine cavity and cervix showed no special change. Tubes and ovaries were unnoteworthy. Esophagus, stomach, and intestines gave negative findings, except a segment, about 100 cm. proximal to the ileocecal valve, which exhibited a peculiar honey-brown color not due to fecal staining or circulatory block, and not explained by observation of the opened gut.

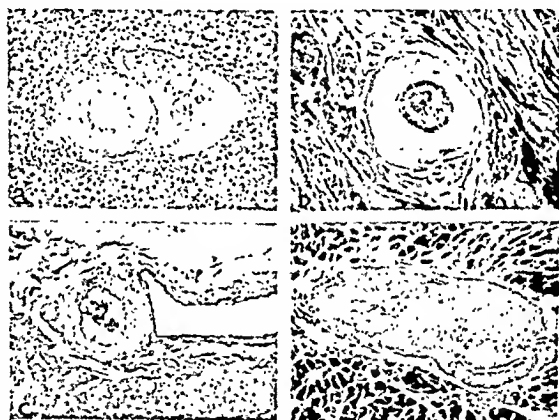


Fig. 1.—Amyloid infiltration of arteries of *a*, liver; *b*, uterus; *c*, adventitia of aorta; *d*, heart.

*Microscopic Examination.*—The findings with the exception of the amyloid change were insignificant, and will only be mentioned incidentally in discussing in detail the more important amyloidosis. Sections were stained with hematoxylin and eosin, Mallory's connective tissue stain, Weigert's elastic stain and van Gieson, Congo red and methyl violet, and sudan III.

The liver was normal, except for amyloid infiltration involving branches of the hepatic artery; these were uniformly amyloid throughout. The hyaline appearance of the arteries was in contrast to the branches of the portal vein which were entirely uninvolved (Fig. 1*a*). In the spleen the pulp was congested, and the Malpighian follicles were inconspicuous. The amyloid change here was the slightest, and consisted only of partial involvement of the central arterioles of the follicles; even these responded poorly to the Congo red and methyl violet stains. The pancreas showed no amyloid whatever in the vessels or elsewhere. The kidneys likewise contained no amyloid in any part of many sections examined. There was a minimum amount of cortical sclerosis and moderate congestion of the glomerular and intertubular capillaries, but no capsular thickening and no hyalinization of the arterioles. There was some degeneration of the tubular epithelium, but no fat reaction with sudan III. There was slight proliferation of the endothelium of the glomerular capillaries. Sections of the intestine from the discolored segments showed necrosis involving the entire wall. The mucosa presented only smudged shadows of the epithelium and lymphoid tissue. The

submucosa still showed structural integrity of the small arteries and veins; the former were amyloid and the latter were unaffected. These intestinal arterioles stained perfectly for amyloid with Congo red and methyl violet. Sections of the subserous myoma of the uterus showed some hyalinization of the fibromyomatous tissue, but these did not react to amyloid stains. All arteries in the section, however, were amyloidized with extreme thickening in some vessels almost completely occluding the lumen (Fig. 1b). The veins again were uninvolved. There was a very small amount of amyloid in the tissues about some of the arteries.

In the heart there was seen a small amount of necrosis and fibrosis in the subendocardial fields, involving especially the papillary muscles, but there was no amyloid here or elsewhere, except in the walls of the small arteries of the parenchyma (Fig. 1d) and the epicardium, and these were uniformly involved. Again there was contrast in the freedom of the veins from amyloid change. In the aorta the arterioles of the adventitia showed amyloidosis, and the veins were free (Fig. 1c).

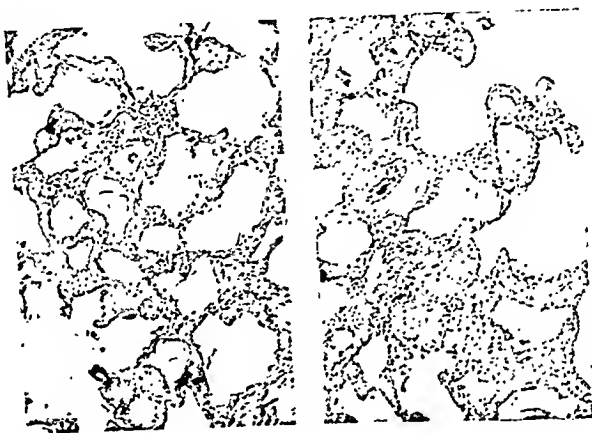


Fig. 2.—Amyloid infiltration of the pulmonary alveoli.

The lungs showed extreme amyloidosis. In addition to the practically universal involvement of both arteries and veins there was almost complete amyloid infiltration of the alveolar walls (Fig. 2). The great thickening of the vessel walls thus induced reduced the lumen markedly and in the smaller vessels to a minimum (Fig. 3). The amyloid of the alveolar walls was presumably related to the capillary wall, but the thickening was relatively so great, and various nodular enlargements so numerous, that fine distinctions could not be made. It is difficult to conceive of much function in lungs so altered. The bronchioles were inconspicuous with lining epithelial cells desquamated and lumina compressed by the swelling of the neighboring vessel walls. There was no amyloid in the bronchial walls. In the few fields where the alveolar walls showed little amyloid, the alveolar spaces contained monocytic cells, but there was no inflammatory exudate or edema. The only demonstrable lesion in the lungs was amyloidosis, and this was diffuse and almost complete. The response to amyloid stains (Congo red and methyl violet) was perfect.

In sections of the various organs stained with Weigert's elastic stain, it was noted that the site of amyloid formation was apparently primarily the media, later extending to the intima. This was well seen in vessels of the uterus and lungs. The intimal involvement tended to close the lumen, while the medial lesions thickened the wall and distended the adventitia but did not seem to involve it.

When the nature of lesions in this case was apparent, we looked for, and fortunately were able to find, the paraffin blocks made from the biopsies of the eyelid and groin done four months previously. Sections of the skin of the eyelid showed a thin epidermal layer, and immediately beneath it and extending through the entire section clumps and masses of tissue containing one or more vessels of capillary or precapillary size. These tissue masses looked like hyalinized or relatively acellular fibrous tissue, but they took the amyloid

stains throughout (Fig. 4a). The vessel walls did not take any amyloid stain. A similar appearance and reaction was noted in the sections of the skin of the groin. In one field of these sections were arterioles, showing hyalinized walls quite like those in the same vessels in the viscera. These vessels, however, did not give the amyloid reaction as did the extravascular homogeneous tissue in the eyelid sections (Fig. 4b).

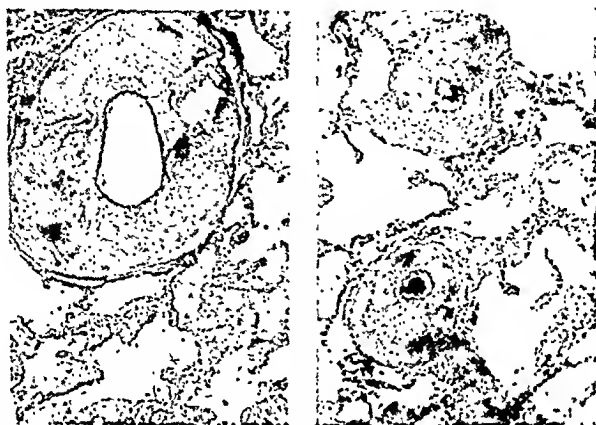


Fig. 3.—Amyloidosis of the pulmonary vessels. Note the marked narrowing of the lumen.

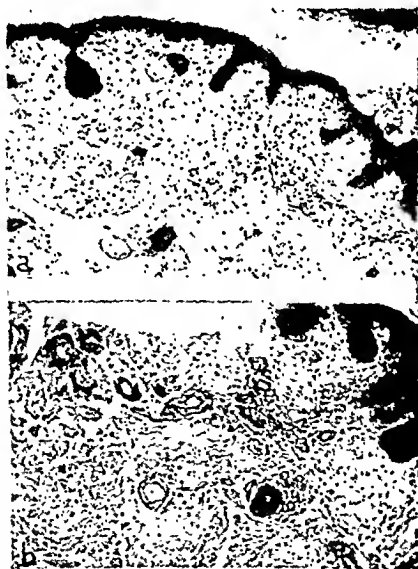


Fig. 4.—a, Skin of eyelid showing amyloid in corium and subcutaneous tissues. b, Skin of groin showing hyalinization of vessel walls and amyloid of the mesodermal tissues.

The final pathologic diagnosis was primary systemic amyloidosis with maximum concentration in the lungs, moderate involvement of the skin, and involvement limited to the small and medium-sized arteries systemically. Moderate cardiac hypertrophy.

#### COMMENT

Here is a case of primary amyloidosis with lesions in the lung quite as great quantitatively as those found in a tuberculous or pneumonic infection of the organ, and yet unrecognized clinically as have been the great majority of atypic

amyloidoses in the past. The failure of the clinical diagnosis in this condition is probably dependent upon (a) the retention of the old conception of amyloid disease as exclusively a secondary condition with massive lesions in the spleen and liver; (b) the rarity of the atypic forms; (c) the variations in the dominant lesions and consequent clinical phenomena in primary amyloidosis; (d) unfamiliarity with diagnostic leads, such as the condition of the tongue; (e) the failure to be amyloid conscious with respect to skin lesions.

The rarity of primary amyloidosis is indicated in Reimann's<sup>2</sup> statement in 1935 that up to that time about 35 cases had been reported. Reimann's case, reported at that time, is apparently the first to be diagnosed clinically. Of these 35 cases, 17 were systemic with general distribution of the amyloid; the remaining were primary, with the localization of the amyloid largely in one or two organs. Using this classification, Koletsky and Stecher<sup>3</sup> in 1939 were able to find only 23 cases of primary systemic amyloidosis in the literature, and added one of their own. Most of the cases have been reported since 1929, when Lubarsch's<sup>4</sup> article aroused interest in the condition. Very few of them have been reported in medical journals dealing with general or internal medicine: most of them have appeared in publications devoted to pathology or dermatology, all of which helps to account for the general unfamiliarity with the subject. Atypic or primary amyloidosis should not be confused with cases of secondary amyloid presenting lesions in unusual situations, as in multiple myeloma. Nor should cases of atypic amyloidosis be considered good examples of the primary form when presenting massive lesions of the spleen, liver, or kidneys, in addition to deposits elsewhere.

In the cases of primary localized amyloidosis there is considerable variation in the distribution of the amyloid deposit, so that the concentration may be dominant in one or two organs or parts, such as the tongue, heart, skin or lungs, and less frequently in the gastrointestinal tract, female genitals, joints, muscle, bone marrow, or even the nervous system. Though such cases are reported as atypic, local, or primary amyloidosis of the heart,<sup>4</sup> lung,<sup>5</sup> skin,<sup>6</sup> or tongue,<sup>7</sup> there may be actually a systemic or diffuse distribution of amyloid scant enough in other parts to be only recognized by the microscope, as in the case reported here. Few cases in the literature show the dominant pulmonary lesion seen in our case. In either the local or systemic form of primary amyloidosis the tissue affected is mesodermal and usually intimately related to the vascular system.

Primary amyloidosis is slowly coming into recognition as a clinical entity, and ante-mortem diagnoses of the condition, now relatively few, may be increased by attention to certain clinical phenomena. Unexplained fatigue and dyspnea are common nonspecific symptoms marked in major involvement of the heart or lungs. Gastrointestinal complaints may actually be amyloid in nature, as may be joint symptoms. The leads which may indicate the specificity of other symptoms and make the diagnosis possible are found in the tongue and skin.

Involvement of the tongue is one of the most common and most suggestive signs of atypic amyloidosis. This organ was the seat of amyloid deposit in 20 of 24 cases reported by Koletsky and Stecher.<sup>3</sup> The macroglossia may be

so great as to cause dysphonia and dysphagia, and even lead to the diagnosis of malignancy, as in one of Lubarsch's<sup>1</sup> cases which was diagnosed as carcinoma of the tongue. In Gerstel's<sup>2</sup> case the swelling was so great that the patient could not close her mouth, and sarcoma of the tongue was suspected. Warren,<sup>3</sup> in a discussion of a case reported by Robertson and Brunsting,<sup>10</sup> cites two cases and states that the patients in both instances complained that their tongues were too large for their mouths and that they had difficulty in swallowing. Their tongues had the characteristic rubber feel of amyloid spleen or liver. Warren was able to make a diagnosis in the second case from the tongue lesion. The part may be deeply furrowed and fissured. Our patient made no complaint at any time referable to the tongue, and we unfortunately did not examine tongue sections after death. It is not unlikely that had we done so we would have found amyloid. Robertson and Brunsting's<sup>10</sup> case did not have symptoms referable to the tongue, but showed amyloid involvement post mortem.

Amyloid lesions of the skin are not as common as those of the tongue, and amyloid skin lesions may be of the purely local type and not indicative of systemic amyloidosis. Schilder<sup>11</sup> examined post mortem the skin of 14 patients showing advanced secondary amyloidosis and was able to demonstrate amyloid deposits in seven, including amyloid lesions in the sweat and sebaceous glands. The skin, however, showed no clinical change. When a case presents an obscure clinical complex and skin lesions are found, they may be of the utmost diagnostic importance, especially in conjunction with tongue symptoms. Amyloid skin lesions are described<sup>12</sup> as small papules, yellowish, yellowish brown, or brown in appearance. In our case they were yellowish brown and somewhat nodular. The injection of Congo red intravenously in cases of primary amyloidosis has not yet been properly evaluated. The test was negative in the cases of von Bonsdorff,<sup>13</sup> Reimann,<sup>2</sup> and Nomland.<sup>6</sup> Nomland<sup>6</sup> has injected Congo red subcutaneously or intrautaneously in the region of the lesions with success, and his results have been confirmed by Robertson and Brunsting,<sup>10</sup> and Philpott and Freshman.<sup>14</sup> In such injections the first diffuse red staining of the skin and lesions later fades, leaving the amyloid lesions a rose red, and this may persist for days or even months. The specific staining of the amyloid lesions with Congo red was later proved by biopsy in Nomland's<sup>6</sup> case. This test, if reliable, should prove of great diagnostic value.

Finally, the diagnosis may be definitely made by biopsy of the skin or glossal lesions. Such sections usually give typical amyloid reactions with methyl violet and other specific stains. The importance of the amyloid-conscious state in such examinations is evident, however, as is seen by the failure of three pathologists to recognize the amyloid in our case, and the fact that the amyloid nature of the sections was not realized in Koletsky's and Stecher's<sup>3</sup> case until the third biopsy had been done.

#### SUMMARY

A case of primary amyloidosis is reported in which the distribution of amyloid was systemic but massive only in the lungs, and to some extent the skin. The condition is rare and is seldom diagnosed clinically. The diagnostic importance of lesions of the tongue and skin is stressed.

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## EXPERIMENTALLY INDUCED HEMATOPOIESIS IN ANEMIC RABBITS\*

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**H**EMORRHAGE is a well-recognized stimulus of hematopoiesis. The following experiment was accordingly devised to determine whether or not posthemorrhagic hematopoiesis is due to a stimulant circulating in the blood of animals subjected to a single large hemorrhage.

### MATERIAL AND METHODS

Fifteen healthy rabbits of comparable age and weight were kept under observation for a week or longer. During this time three or four determinations were made of their erythrocyte and leucocyte counts and hemoglobin percentages. These varied only slightly and were within the accepted limits of normal.<sup>1</sup> The average of the values obtained for each rabbit was accordingly accepted as the "normal" for that animal. Following this preliminary period of observation, the rabbits were rendered anemic by removing blood from the heart. The erythrocyte and hemoglobin values in each case were reduced as rapidly as possible to about 60 per cent of the normal, and maintained by repeated bleeding at approximately this level for variable periods. The amount of blood removed and the frequency of bleeding varied considerably, the erythrocyte counts and hemoglobin value being the sole determining factors in each case. After the anemia had been maintained for ten to fourteen days, the animals were divided into three groups, as follows: (1) A control group, comprising 4 rabbits, received no transfusions. (2) A second control group of 5 rabbits received transfusions intravenously with blood removed by cardiac puncture under sterile conditions from healthy rabbits. (3) A group of 6 rabbits received transfusions from "prepared" donors. Preparation of the donors consisted of preliminary bleeding in order to stimulate hematopoiesis. The bleeding was performed by cardiac puncture, from 15 to 20 c.c. of blood being removed by a single puncture, about three to five days before it was planned to use the blood of these rabbits for transfusion purposes. The resulting degree of hematopoietic stimulation was judged by repeated reticulocyte counts. Blood was removed from the stimulated donors by cardiac puncture and injected intravenously into the anemic rabbits when the reticulocyte response of the donors was judged to be at its peak. (Subsequent reticulocyte counts on the stimulated donors showed that the peak was judged correctly in all but one instance. The donor in this case showed a moderate increase of its

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reticulocytes the day after its blood was used for transfusion.) A distinct reticulocytosis occurred in all the prepared donors. Determinations were made of the reticulocytes, erythrocytes, leucocytes, and hemoglobin at frequent intervals until the erythrocyte counts were at or above normal.

All the rabbits used in this experiment received the same general care. They were housed in well-aired metal cages in a large, well-ventilated animal room, and allowed a balanced commercial ration and water *ad libitum*.

TABLE I

SUMMARY OF EFFECTS ON ERYTHROCYTES AND HEMOGLOBIN OF RABBITS COMPOSING THREE EXPERIMENTAL GROUPS

| EFFECTS                                     | NORMAL<br>CONTROLS | RABBITS GIVEN TRANS-<br>FUSIONS FROM |                    |
|---|--------------------|--------------------------------------|--------------------|
|   |                    | HEALTHY<br>DONORS                    | PREPARED<br>DONORS |
| <i>On Erythrocytes</i>                      |                    |                                      |                    |
| Number of animals                           | 3.0 <sup>a</sup>   | 5.0                                  | 6.0                |
| Average duration of anemia, days*           | 13.7               | 11.0                                 | 11.5               |
| Average degree of anemia, per cent†         | 56.5               | 58.4                                 | 60.0               |
| Average total amount of blood removed, c.c. | 175.0              | 140.0                                | 185.0              |
| Average amount of blood transfused, c.c.    |                    | 38.0                                 | 33.0               |
| Average recovery time, days‡                | 18.0               | 10.8                                 | 8.0                |
| <i>On Hemoglobin</i>                        |                    |                                      |                    |
| Number of animals                           | 3.0 <sup>a</sup>   | 3.0 <sup>b</sup>                     | 5.0 <sup>c</sup>   |
| Average duration of hemoglobinemia, days    | 10.3               | 14.0                                 | 10.6               |
| Average degree of Hb. deficiency, per cent† | 61.0               | 68.0                                 | 63.0               |
| Average total amount of blood removed, c.c. | 175.0              | 152.0                                | 177.0              |
| Average amount of blood transfused, c.c.    |                    | 46.0                                 | 37.0               |
| Average recovery time, days‡                | 19.2               | 9.0                                  | 6.8                |

\*The average duration of the anemia was determined by computing the time in days during which there was a severe deficiency of erythrocytes and hemoglobin.

†The average degree of anemia was computed by determining the period during which each animal showed its most severe erythrocyte and hemoglobin deficiency.

‡The average recovery period represents the time taken for the restoration of the erythrocytes and hemoglobin to normal values.

<sup>a</sup>One control rabbit died before the erythrocyte count and hemoglobin returned to normal and is, therefore, not included in the summary.

<sup>b</sup>The hemoglobin of two rabbits in this group remained subnormal during the experimental period. They are omitted from the summary.

<sup>c</sup>One rabbit in this group still had a hemoglobinemia when the experiment was discontinued. It is not included in the summary.

## RESULTS

The individual protocols are illustrated graphically in Charts 1 to 6. These show the amount of blood removed, the resultant degree of anemia, the amount of blood transfused, and the recovery period for each rabbit. The untransfused control group recovered very slowly and imperfectly, as judged by both the erythrocyte and hemoglobin determinations. One animal in this group died six days after the last bleeding. The day before death its erythrocyte count was still only 40 per cent of the pre-experimental level. At autopsy it showed severe anemia, dehydration, and emaciation. Another rabbit in this group required a month for the restoration of its erythrocyte count to normal. During all this time it was in poor condition. The remaining two in this group recovered in ten and sixteen days, respectively. It is interesting that neither the total amount of blood removed nor the frequency of bleeding influenced decisively the time required for recovery, since Rabbit No. 114 recovered the most rapidly, in spite of having been bled more severely than any of the other

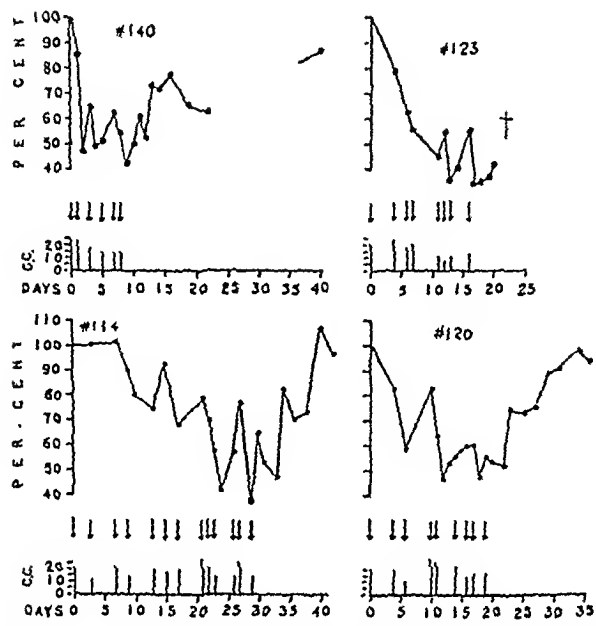


Chart 1.—Control group, not given transfusions. Erythrocyte counts, expressed in percentage of the normal. Arrows pointing downward represent bleeding from the heart.

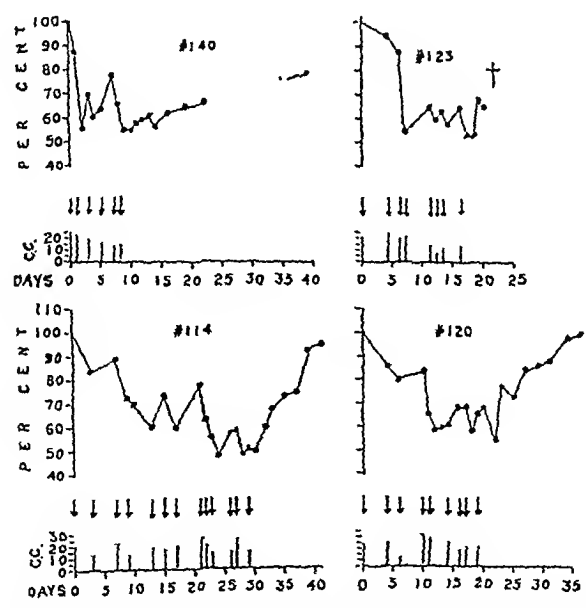


Chart 2.—Control group, not given transfusions. Hemoglobin, expressed in percentage of the normal. Arrows pointing downward represent bleeding from the heart.

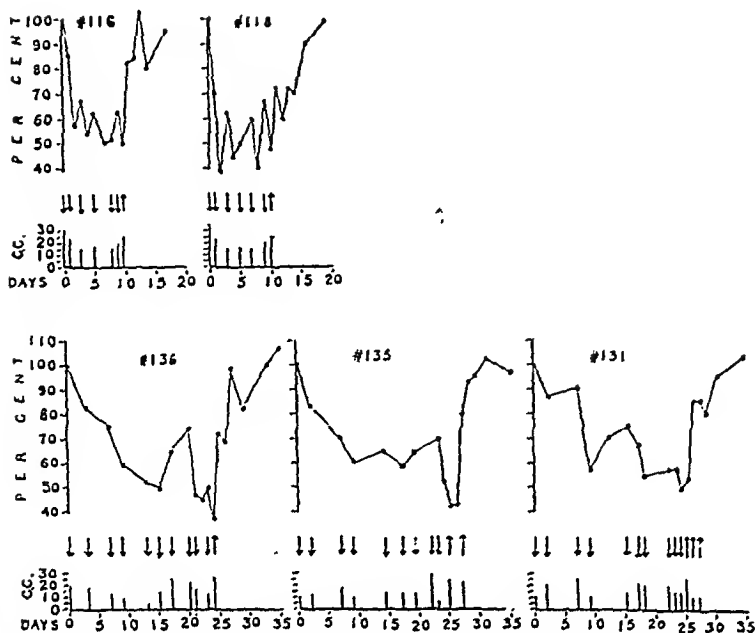


Chart 3.—Control group, given transfusions from healthy donors. Erythrocyte counts, expressed in percentage of the normal. Arrows pointing downward represent bleeding from the heart. Arrows pointing upward represent intravenous transfusion.

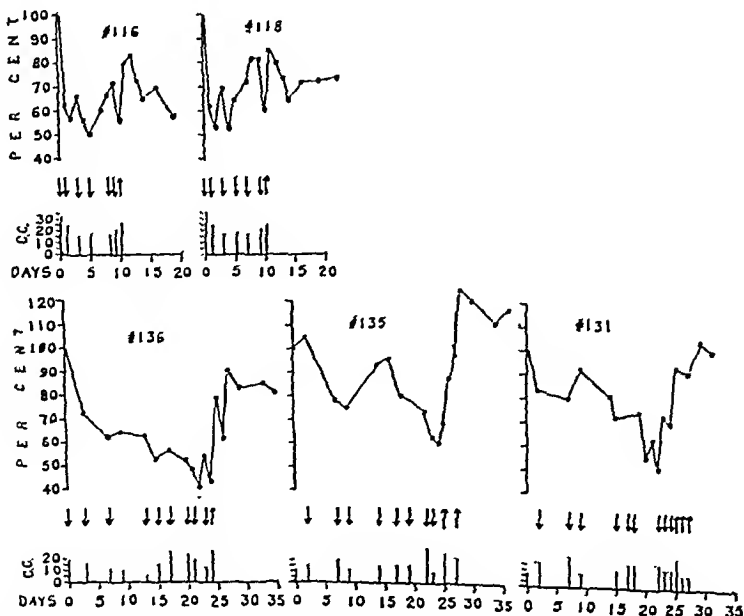


Chart 4.—Control group, given transfusions from healthy donors. Hemoglobin, expressed in percentage of the normal. Arrows pointing downward represent bleeding from the heart. Arrows pointing upward represent intravenous transfusion.

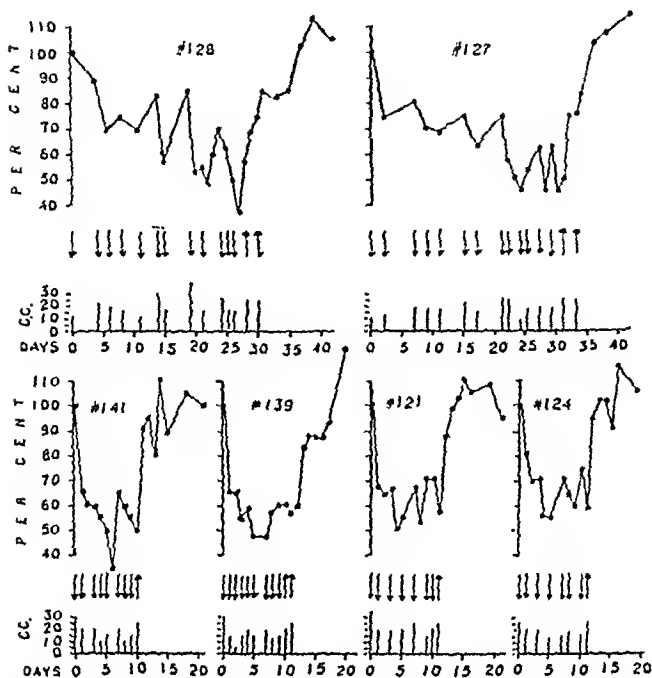


Chart 5.—Experimental group, given transfusions from "prepared" donors. Erythrocyte counts, expressed in percentage of the normal. Arrows pointing downward represent bleeding from the heart. Arrows pointing upward represent intravenous transfusion.

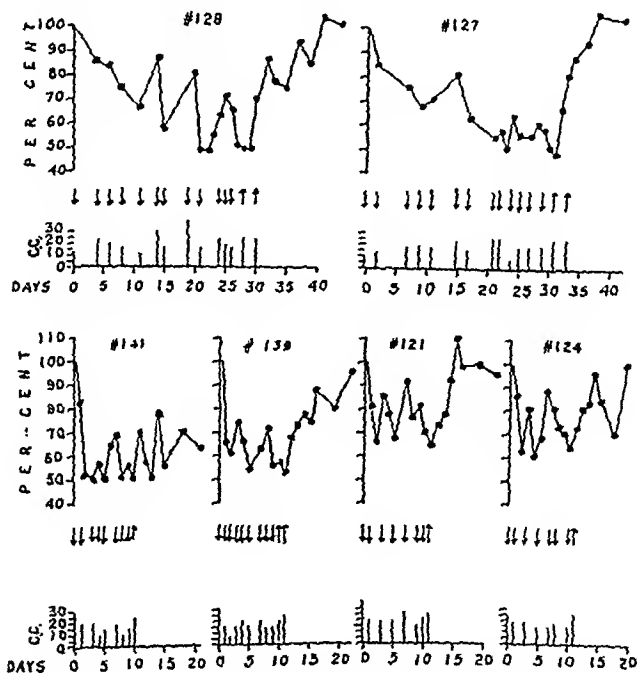


Chart 6.—Experimental group, given transfusions from "prepared" donors. Hemoglobin, expressed in percentage of the normal. Arrows pointing downward represent bleeding from the heart. Arrows pointing upward represent intravenous transfusion.

rabbits in this group. This was true with respect to the restoration of both the erythrocyte and the hemoglobin values. The average recovery period for this group was eighteen days for the erythrocytes, and 19.3 days for the hemoglobin.

The second control group, transfused from healthy donors, recovered more rapidly than the group receiving only routine care (Table I). The erythrocyte recovery period for all 5 rabbits composing this group averaged 10.8 days, recovery being uninterrupted. The immediate hemoglobin recovery period was complete in only 3 of the 5 animals composing this group, and averaged nine days. All 5 survived the experiment and showed definite clinical improvement the day after one transfusion, with complete clinical recovery within a week after the termination of the experiment.

The rabbits transfused from stimulated donors recovered in an average of eight days with respect to their erythrocyte counts. All but one showed a satisfactory restoration of the hemoglobin content of the red blood cells within an average period of 6.8 days. The clinical recovery of the rabbits in this group was slightly more prompt than that of the group transfused from healthy donors.

The reticulocyte counts of all rabbits varied considerably. In general, there was a distinct reticulocytosis in all rabbits after the first bleeding. This declined irregularly after the third or fourth hemorrhage. A secondary increase in the reticulocytes appeared in all three groups when the bleeding was discontinued, although it was later in the untransfused group than in the two transfused groups. No significant difference in the degree or in the promptness of this secondary reticulocytosis was noted in the two transfused groups.

#### COMMENT

The number of animals used is too small to permit the drawing of sweeping conclusions. There is justification for the general statement that the rabbits receiving blood from stimulated donors recovered more rapidly and satisfactorily than either of the control groups. This is borne out both by the data summarized in Table I and by the clinical behavior of the animals. The two groups receiving transfusions gained weight and strength much more rapidly than the group receiving only routine care, and the group given transfusions from prepared donors improved more rapidly than the group given transfusions from healthy donors. There was some parallel in the restoration of the erythrocytes and that of the hemoglobin, but the restoration of the latter, with respect to the individual animals in each group, was less constant and returned more slowly to normal levels.

The question arises whether the erythrocytes of the "stimulated donors," being relatively young, may have been more viable than those of the healthy donors, thus accounting to some extent for the more rapid recovery of the rabbits in the one group. This does not seem a reasonable explanation, because the total amount of blood injected was comparatively small. Furthermore, the three rabbits in this group receiving only one transfusion recovered about as promptly as the three rabbits given transfusions on two occasions.

It may appear anomalous that a hematopoietic stimulant was apparently not formed in the rabbits rendered anemic by repeated bleeding. However, it is a well-confirmed observation in man that repeated hemorrhages are unaccompanied by evidences of regeneration of erythrocytes, whereas a single hemorrhage is succeeded by evidences of erythropoiesis (reticulocytosis and polychromatophilia). The reason for this is not evident. It is worth emphasis that the bone marrow of rabbits has an enormous "reserve," as judged by the large number of fat cells normally present in rabbit bone marrow.<sup>2</sup> These, presumably, are capable of metaplasia with transformation into hematopoietic foci.

Additional experiments along similar lines have been planned. The present experiment is being reported now because it is felt that clinical application may be made of the method of "donor stimulation"; that is, both on theoretical and experimental grounds, the conclusion seems justified that blood from human donors, shortly after a small phlebotomy, may act as a hematopoietic stimulant if administered to patients with hypoplastic, aplastic, or "refractory" anemia.

#### SUMMARY

1. Fifteen rabbits rendered anemic by repeated bleeding from the heart were divided into three groups.

2. One group received only routine care. A second group received routine care and intravenous transfusions from healthy donors. The third group was given routine care plus intravenous transfusions from donors previously stimulated by the removal of a small quantity of blood from the heart.

3. The two groups given transfusions improved more rapidly, both clinically and hematologically, than the group receiving no transfusions.

4. The group receiving transfusions from prepared donors improved more rapidly, both clinically and hematologically, than either of the two control groups.

5. It is suggested that preliminary phlebotomy of human donors may provide an effective method of treating hypoplastic, aplastic, and refractory anemia in man.

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# CLINICAL AND LABORATORY INVESTIGATIONS ON THE EXTRACT OF THE EUROPEAN MOUNTAIN ASH BERRY, WITH PARTICULAR REFERENCE TO ITS ANTIHEMORRHAGIC ACTIVITY\*

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THE berry of the European mountain ash, *Sorbus aucuparia* L., has reportedly been used as a "spring tonic" by the Laplanders of the northern Scandinavian countries for many years.<sup>11</sup> Traditionally, the products obtained from the berry are consumed in the spring in the belief that they have some beneficial action, particularly on the liver. It has been suggested that the change from the high fat diet of winter to one containing more green vegetables in the spring, results in some type of biliary stasis. Folklore led to the assumption that the berry acted as a "flushing agent."

The United States Dispensatory<sup>9</sup> describes the *Sorbus* fruit as having been "used in scurvy, and in infusion, as a remedy in hemorrhoids and strangury." It further states that the fruit contains a nonfermentable monosaccharide, sorbinose,  $C_6H_{12}O_6$ , isomeric with levulose; the crystalline saccharine principle, sorbitol,  $C_6H_{14}O_6$ , isomeric with mannitol; sorbic and parasorbic acids,  $C_6H_8O_2$ ; malic acid  $C_4H_6O_5$ . The seeds of the fruit contain 22 per cent of fixed oil. The Biologic Research Division, Philadelphia College of Pharmacy and Science,<sup>2</sup> has confirmed these analyses and in addition has reported the definite presence of appreciable amounts of ascorbic acid.

A clinical and laboratory investigation of the *Sorbus* berry as to its mode of action and possible therapeutic value in hepatobiliary disease has been undertaken. In this paper are described experiments conducted to determine the antihemorrhagic activity of the extracts of this fruit.

## EFFECT ON THE COAGULATION TIME IN HEMORRHAGIC CHICKS

**Method.**—Since the existence of vitamin K deficiency was first suspected in chicks by Dam<sup>3</sup> in 1929, the chick has been employed in the assay of the natural and synthetic antihemorrhagic substances. The biological assay method of vitamin K employed in this study was that of Ansbacher.<sup>1</sup> The clotting time was determined as described by this author, except that larger test tubes (1 by 7.5 cm.) were used. Ration K-1 was fed to all the test animals. Although the quantitative determination of the plasma prothrombin level would have been preferable to the gross clotting time, attempts to apply both the method of Quick<sup>5</sup> and that of Smith<sup>7</sup> to the small volume of blood obtainable from chicks were without success.

\*From the Gallbladder Clinic, a joint project of the Departments of Medicine, Pathology, and Surgery, and the Laboratories of the University Hospital, Ohio State University College of Medicine.

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*Results.*—In an attempt to produce the hemorrhagic syndrome typical of avitaminosis K, 920 chicks were placed on Aushbacher's basal ration K-1.<sup>2</sup> The syndrome developed more often in the White Rock than in the White Leghorn chicks. Some animals on this diet retained normal coagulation time for periods as long as three weeks. This emphasized the necessity of a freshly prepared diet. Ration K-1 contains 17.5 per cent of ether-extracted fish meal. Since putrified fish meal is known to be an excellent source of vitamin K<sub>2</sub>, great care must be exercised to minimize bacterial action in this basal diet.

The alcoholic extract of the dried berry (Sorparin) was not sufficiently concentrated to be administered satisfactorily to the animals. This alcoholic extract was, therefore, subjected to rapid extraction with petroleum ether (b. p. range, 30° to 60° C.); 1 Gm. of this latter extract represented approximately 20 Gm. of Sorparin or 50 Gm. of the dried berry. Furthermore, the dried Sorbus berry was ground and extracted in a similar manner; 1 Gm. of this extract was equivalent to approximately 40 Gm. of the original material.

TABLE I  
BIOLOGICAL ASSAY

| EX-<br>PERI-<br>MENT | SUBSTANCE                               | DOSE     | NUM-<br>BER<br>OF<br>CHICKS | AVERAGE BLOOD<br>CLOTTING TIME   |  |
|----------------------|---|----------|-----------------------------|----------------------------------|--|
|                      |   |          |                             | BEFORE<br>TREATMENT<br>(MINUTES) | AFTER<br>24-HOUR<br>TREATMENT<br>(MINUTES) |
| A                    | 2-methyl-1,4-naphthoquinone             | 0.25γ    | 5                           | greater than 30                  | 6 (1-15)                                   |
|                      | 2-methyl-1,4-naphthoquinone             | 0.50γ    | 6                           | 30                               | 2 (1-4)                                    |
|                      | Petroleum ether extract of Sorparin     | 20.0 mg. | 5                           | 30                               | 2 (1-3)                                    |
|                      | Petroleum ether extract of Sorparin     | 10.0 mg. | 5                           | 30                               | 2 (1-3)                                    |
|                      | Sorparin                                | 75.0 mg. | 6                           | 30                               | 5 (2-8)                                    |
| B                    | 2-methyl-1,4-naphthoquinone             | 0.25γ    | 8                           | 30                               | 10 (2-90)                                  |
|                      | 2-methyl-1,4-naphthoquinone             | 0.50γ    | 8                           | 30                               | 2 (1-4)                                    |
|                      | Petroleum ether extract of Sorparin     | 20.0 mg. | 8                           | 30                               | 1 (½-3)                                    |
|                      | Petroleum ether extract of Sorparin     | 10.0 mg. | 8                           | 30                               | 2 (¾-4)                                    |
| C                    | 2-methyl-1,4-naphthoquinone             | 0.50γ    | 8                           | 30                               | 3 (1-4)                                    |
|                      | Petroleum ether extract of Sorparin     | 20.0 mg. | 6                           | 30                               | 1 (½-2)                                    |
|                      | Petroleum ether extract of Sorparin     | 10.0 mg. | 6                           | 30                               | 2 (1-3)                                    |
|                      | Petroleum ether extract of Sorbus berry | 40.0 mg. | 5                           | 30                               | 4 (1-9)                                    |
|                      | Petroleum ether extract of Sorbus berry | 20.0 mg. | 6                           | 30                               | 18 (2-90)                                  |

The experiments reported in Table I are those in which there was the successful production of the hemorrhagic syndrome and in which sufficient depression of the clotting time was obtained for approximate comparison with the positive synthetic vitamin K controls. The extracts were administered in 0.1 c.c. amounts of cod-liver oil to 17-day-old White Rock chicks on ration K-1. After twenty-four hours of the test period blood clotting time determinations were made.

Experiment D was performed in an entirely different manner: 52 one-day-old White Rock chicks were placed on Ration K-1. After eleven days the blood clotting time on six chicks averaged eighteen minutes. The remaining chicks were separated into two groups of 23 each. Group 1 was continued on ration K-1 *ad libitum*. Group 2 was fed ration K-1 with a known amount of dried Sorbus berry added. Seven days later blood clotting times were taken on these



18-day-old chicks. The average clotting time of the control group 1 was sixty-two minutes (23 chicks: range four to ninety minutes). The average clotting time in group 2, fed dried berries, was two and one-half minutes (23 chicks: range one to eleven minutes). Two hours after the clotting times were taken, the mortality in group 1 was 35 per cent, in group 2, 5 per cent. During the seven-day test period the 23 chicks in group 2 consumed 300 Gm. of the dried *Sorbus* berry. Blood, bile, and livers were obtained for study. Our preliminary biochemical studies show a marked increase in the neutral fat content of the livers in group 1 in contrast with group 2, which was normal.

The results obtained in this preliminary investigation must be regarded as qualitative or, at the most, semiquantitative. It is apparent from the data of Table I that there is present at least one Ansbaeher unit<sup>1</sup> of vitamin K potency in 10 mg. of the crude petroleum ether concentrate of Sorparin. This may be further interpreted to mean there are more than 5 units present in one gram of Sorparin, or the minimum of 2 units in one gram of the dried berry of the European mountain ash. Quantitative biological assays are now being carried out in our laboratory to determine the optimum potency of this drug with highly refined concentrates.

#### EFFECT ON THE PLASMA PROTHROMBIN LEVEL IN MAN

The 14 cases to be reported here include those patients on Sorparin therapy alone, or those with other medications, on whom prothrombin determinations were done before and after treatment.

*Method.*—Quick's<sup>2</sup> prothrombin technique was employed throughout this study. The results obtained by this relatively accurate, rapid, and simple procedure in more than 2,000 tests in our laboratories have been generally satisfactory only when carried out with strict adherence to the following: (a) the careful preparation and standardization of thromboplastin; (b) the use of "proved" normal controls; and (c) the titration of undiluted and diluted concentrations of freshly obtained plasma or plasma preserved by freezing.

The drug was administered orally in tablets containing 3 grains of the solid alcoholic extract of the European mountain ash berry. Three grains of this extract is equivalent to approximately 7.5 grains of the dry berry.

*Results.*—The data on 8 of the 14 cases studied are presented in Table II. The data on the remaining 6 cases on which more intensive observations were made are recorded in Graphs 1 to 6. Table III is a summary of the results obtained in all 14 cases.

The results of this investigation on the prothrombin response to Sorparin medication present several interesting observations. Irrespective of the diagnosis in each of the 14 cases studied, in only one (Case 31, Graph 5) was the prothrombin level diminished further after Sorparin medication. In this particular case (biliary dyskinesia with persistent fistula) the decrease in prothrombin was noted only when bile acids were administered with Sorparin. When Sorparin was given alone, the prothrombin level rose in each of two periods of medication. From the data summarized in Table III, it may be concluded that the plasma prothrombin showed some decrease in 8 per cent, "no change" in 20 per cent, and a definite increase in 72 per cent of the 25

TABLE II  
PROTHROMBIN LEVEL BEFORE AND AFTER MEDICATION

| CASE NO. | SEX AGE | MEDICATION     |                  |                   | PROTHROMBIN          |                     | DIAGNOSIS                               |
|----------|---------|----------------|------------------|-------------------|----------------------|---------------------|---|
|          |         | DURATION WEEKS | SORPARIN GR./DAY | BILE ACID GR./DAY | BEFORE TREATMENT (%) | AFTER TREATMENT (%) |   |
| 4        | F 23    | 2              | 18               | 10-I.B.S.         | 60                   | 97                  | Chronic cholecystitis                   |
| 6        | F 62    | 1              | 9                | 11½-U.K.C.        | 65                   | 85                  | Cholecystitis; cholelithiasis           |
| 16       | F 51    | 2              | 12               | None              | 75                   | 83                  | Postsurgical biliary syndrome           |
| 26       | F 44    | 1              | 9                | None              | 75                   | 95                  | Cholelithiasis                          |
| a 30     | F 51    | 3              | 18               | None              | 60                   | 63                  | Intermittent common duct obstruction    |
| b 43     | M 57    | 4              | 18               | 15-I.B.S.         | 65                   | 75                  | Hepatitis; syphilis; cholelithiasis (?) |
|          |         | 4              | 9                | None              | 62                   | 65                  | Postsurgical biliary syndrome           |
| a 46     | F 54    | 3              | 18               | 15-I.B.S.         | 57                   | 107                 | Cholecystitis; menopause                |
| b 48     | F 55    | 2              | 18               | None              | 60                   | 62                  |   |
|          |         | 2              | 18               | None              | 34                   | 64                  |   |

Sorparin = Alcoholic extract of the European mountain ash berry.  
I.B.S. = Iron bile salts.

U.K.C. = Unconjugated-ketocholic acids.

a = First period of medication.

b = Second period of medication.

TABLE III  
SUMMARY: PROTHROMBIN RESPONSES TO SORPARIN ALONE AND WITH BILE ACIDS

| DIAGNOSIS<br>(14 CASES)                                 | PROTHROMBIN RESPONSE TO MEDICATION |             |           |                                     |             |           |
|---|------------------------------------|-------------|-----------|-------------------------------------|-------------|-----------|
|   | PERIODS ON SORPARIN ALONE          |             |           | PERIODS ON SORPARIN WITH BILE ACIDS |             |           |
|   | DECREASED                          | "NO CHANGE" | INCREASED | DECREASED                           | "NO CHANGE" | INCREASED |
| Cholecystitis and/or cholelithiasis (7 cases)           | 0                                  | 0           | 4         | 0                                   | 0           | 7         |
| Postsurgical biliary syndrome (2 cases)                 | 0                                  | 2           | 0         | 0                                   | 0           | 1         |
| Intermittent common duct obstruction (1 case)           | 0                                  | 1           | 0         | 0                                   | 1           | 0         |
| Hepatitis (syphilis) (1 case)                           | 0                                  | 1           | 0         | 0                                   | 0           | 0         |
| Carcinoma of pancreas with metastasis to liver (1 case) | 0                                  | 0           | 0         | 0                                   | 0           | 2         |
| Biliary dyskinesia with persistent fistula (1 case)     | 0                                  | 0           | 2         | 2                                   | 0           | 1         |
| "Hypoprothrombinemia" (1 case)                          | 0                                  | 0           | 1         | 0                                   | 0           | 0         |
| Total   | 0                                  | 4           | 7         | 2                                   | 1           | 11        |
| Percentage based on 25 periods of medication            | (0%)                               | (16%)       | (28%)     | (8%)                                | (4%)        | (44%)     |

Decreased = Prothrombin level after medication less than before medication.

"No change" = Prothrombin level increased less than 15 per cent after medication.

Increased = Prothrombin level increased greater than 15 per cent after medication.

periods of medication in 14 cases. "No change" is statistically reported when an increase of less than 15 per cent in the prothrombin level was recorded. On the basis of less rigid requirements, it may be stated that some increase in prothrombin was observed in 92 per cent of the 25 periods of medication of Sorparin with or without bile acids and salts.

In the 7 cases of cholecystitis and/or cholelithiasis there was an elevation in prothrombin following four periods of Sorparin treatment and during seven periods of Sorparin-bile acids treatment. Medication was without appreciable effect relative to prothrombin in hepatitis with syphilis (Case 43, Table II) and in intermittent common duct obstruction (Case 30, Table II). Of the two cases (Cases 16 and 46, Table II) of postsurgical biliary syndrome, a marked elevation in prothrombin was observed only in Case 46, and then when iron bile salts were administered with Sorparin. Apparently in the case of carcinoma of the head of the pancreas with metastasis to the liver (Case 49, Graph 3), there were sufficient functioning liver cells for prothrombin production in response not only to vitamin K, but also to Sorparin with bile acids. Case 50 is diagnosed as "hypoprothrombinemia" for want of a better terminology. The case is that of a healthy medical student with no symptoms or demonstrable pathologic dyscrasia except his persistent low basal prothrombin level. Another study of this patient is presenting similar results.

The question may arise as to the effect of bile acids and salts alone on the prothrombin level. Cases 49, 21, and 22 (Graphs 3, 4, and 6), indicate that no elevation can be attributed to bile acids and bile salts therapy alone. This was observed in 5 other patients not included in this series of 14 cases. The foregoing statements and data submitted cannot necessarily be interpreted to mean that bile acids and salts alone cause either a depression or no increase in prothrombin, since similar results are possible without any medication whatsoever.

#### CASE REPORTS

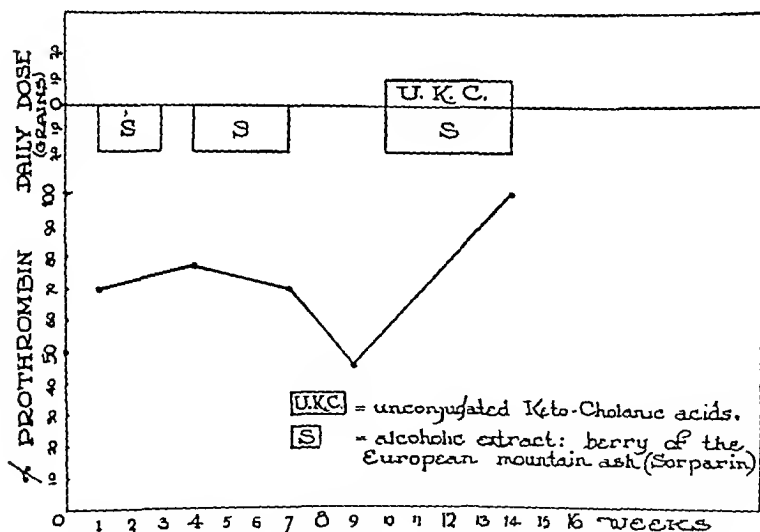
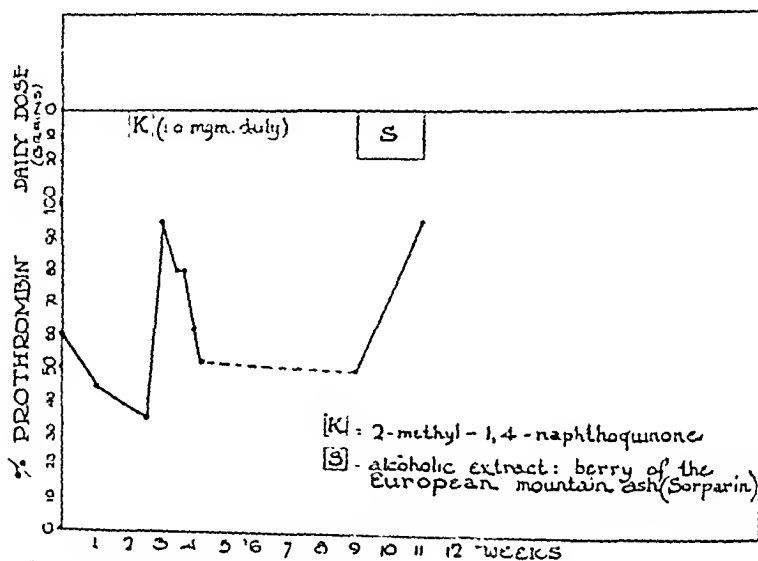
Case abstracts of 6 patients investigated for their prothrombin response to Sorparin medication follow:

CASE 50 (Graph 1).—In a group of approximately 50 normal healthy men and women, a 25-year-old white, male, medical student, was one of only two to show a plasma prothrombin level below 70 per cent. His initial prothrombin of 60 per cent fell to 45 per cent one week later. A bromsulphthalein test was normal. One milligram of synthetic vitamin K daily for three days increased his prothrombin from 36 to 95 per cent. Upon cessation of therapy the prothrombin level fell to 80 per cent in three days, and to 52 per cent in a week. Two weeks of Sorparin treatment (18 grains daily) brought the prothrombin level again to 95 per cent.

CASE 10 (Graph 2).—A white female, aged 60 years, had a history of cholecystitis and cholelithiasis for twenty years. She began to show symptoms of hypertension and of occipital headaches, and her menses ceased. After one month of Sorparin therapy the relief from her biliary symptoms was complete. The basal prothrombin level averaged 45 per cent. When the patient was placed on 18 grains of Sorparin daily, the prothrombin level was between 70 and 80 per cent; on Sorparin with bile acids, it rose to 105 per cent.

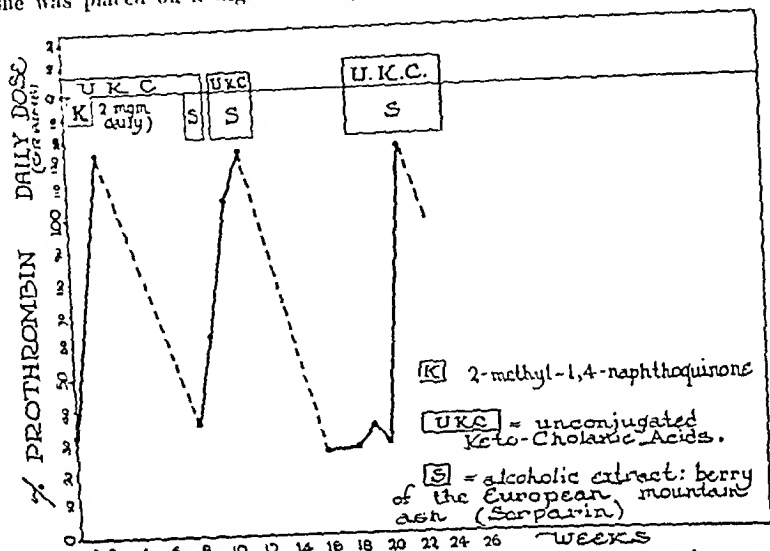
CASE 49 (Graph 3).—A white female, aged 76 years, had jaundice with itching and acholic stools for two weeks. The laboratory data revealed the serum bilirubin 6.7 mg. per cent, hippuric acid 1.35 Gm., nonfilling gall bladder, and prothrombin 32 per cent. Gall bladder drainage showed that the common bile duct was not completely obstructed. Surgery was

refused. A diagnosis of carcinoma of the head of the pancreas was made. Two milligrams of synthetic vitamin K with 7.5 grains of bile acids daily elevated her prothrombin level to 120 per cent in two weeks. Eighteen grains of Sorparin with 7.5 grains of bile acids daily raised the prothrombin level from 35 to 120 per cent, and from 30 to 120 per cent in two subsequent periods of medication. After twenty-two weeks of observation the patient's condition

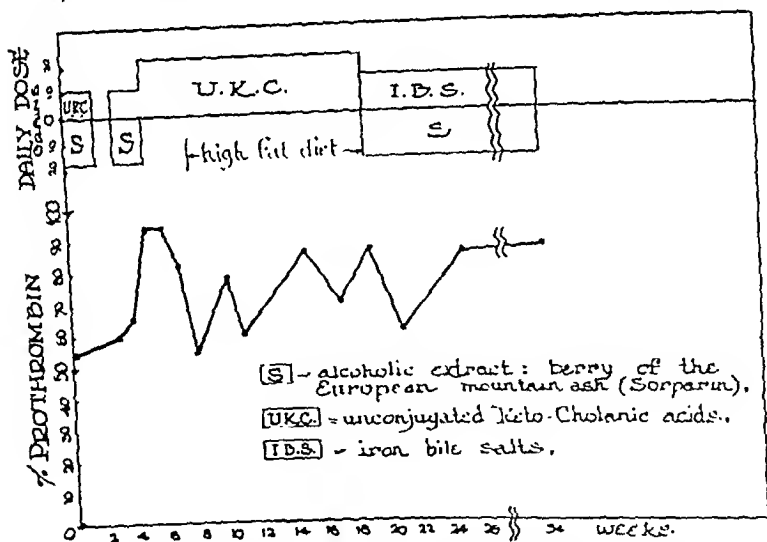


became progressively worse. She died four months later. During the four-month period she was given very little bile acid, vitamin K, or Sorparin. Her prothrombin level averaged 39 per cent. The chief findings at autopsy were mixed squamous-cell carcinoma and adenocarcinoma of pancreas with compression of normal bile duct and metastases to liver and obstructive biliary cirrhosis of liver.

CASE 21 (Graph 4).—A 55-year-old white female had a cholecystostomy. On her first postoperative visit to the Gallbladder Clinic four months later, the prothrombin was 55 per cent. She was given Sorparin and bile acids, and her prothrombin gradually rose to 93 per cent. She was taken off of Sorparin and the bile acid dosage was doubled. In three weeks the prothrombin was down to 55 per cent. The same bile acid therapy was continued, but in addition she was placed on a high fat diet, and the prothrombin level averaged 76 per cent.



Graph 3. CARCINOMA HEAD of PANCREAS (Case #49)

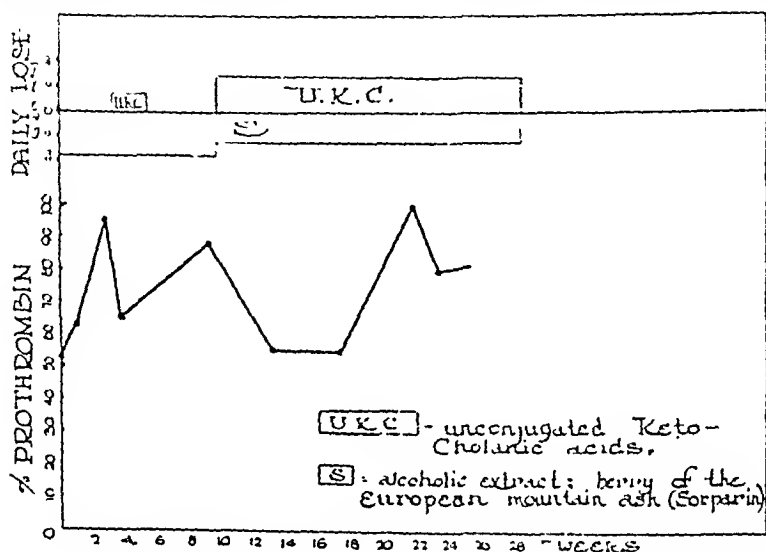


Graph 4. CHRONIC CHOLECYSTITIS; CHOLELITHIASIS (Case #21)

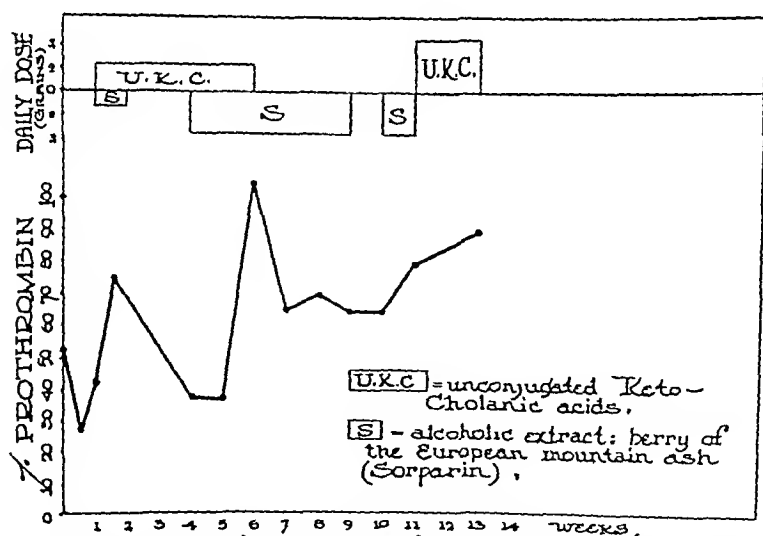
She was then placed on iron bile salts and Sorparin. There was an initial moderate fall in prothrombin followed by an increase (85 per cent). During the thirty-four weeks' study this patient had no complaints referable to the gall bladder.

CASE 31 (Graph 5).—A 72-year-old white female with obstructive jaundice was admitted to the hospital. A cholecystostomy with removal of stones was done, and she slowly recovered. The gall bladder was drained for ten weeks. She was discharged and remained asymptomatic

for four months, when she had an attack of nausea, vomiting, and chills. She was readmitted to the hospital with sharp pain over the upper abdomen, jaundice, acholic stools, and deeply colored urine. Laboratory tests showed a moderately diminished liver function. A cholecystogastrostomy was performed because of a beginning stenosis of the common duct. The patient's convalescence was rather slow, but she was discharged markedly improved. Except for a slight distress in the epigastric region, her condition was excellent and she gained 60



Graph 5. BILIARY DYSKINESIA with PERSISTENT FISTULA (Case #31)



Graph 6. CHOLELITHIASIS (RECURRENT) (Case #22)

pounds. Prothrombin studies were begun at this time. On 18 grains of Sorparin daily the prothrombin level was elevated from 52 to 96 per cent. When bile acids supplemented this therapy, the prothrombin level dropped to 64 per cent. It rose again on Sorparin alone to 90 per cent. The patient was then placed on 12 grains of Sorparin and 15 grains of bile acids daily. The prothrombin level was first decreased to 55 per cent, and then rose to from 80 to 100 per cent.

CASE 22 (Graph 6).—A white female, aged 42 years, had a cholecystostomy with removal of stones in 1936. Her symptoms recurred, and three years later a laparotomy was performed and the gall bladder region was explored. The gall bladder was found imbedded in a dense mass of adhesions, making it impossible to do any operative procedure. Gradually there was a recurrence of nausea, occasional vomiting, and recurrent, transient jaundice. She was admitted for the third time. Laboratory examinations at this time revealed normal serum bilirubin, hippuric acid, and galactose tolerance. However, her prothrombin level was diminished to 52 per cent. She was placed on 6 grains of Sorparin with 11.25 grains of bile acids daily for one week. Her prothrombin increased to 75 per cent. When next seen in the Gall-bladder Clinic one month later, the prothrombin level was 37 per cent, and she was on bile acids only. She was placed on Sorparin along with bile acids, and the prothrombin rose to 105 per cent in two weeks. At this time bile acid therapy was discontinued, and on Sorparin alone she maintained a prothrombin average of 70 per cent.

TABLE IV  
CLINICAL RESULTS OF THERAPY WITH SORPARIN, KETOCHOL, AND BILRON

| NUMBER OF CASES | DIAGNOSIS             | SORPARIN   |               | KETOCHOL   |               | BILRON     |               |
|-----------------|-----------------------|------------|---------------|------------|---------------|------------|---------------|
|                 |                       | RELIEF (%) | NO RELIEF (%) | RELIEF (%) | NO RELIEF (%) | RELIEF (%) | NO RELIEF (%) |
| 22              | Chronic cholecystitis | 72         | 28            | 70         | 30            | 77         | 23            |
| 13              | Cholelithiasis        | 86         | 14            | 72         | 28            | 85         | 15            |
| 2               | Cholecystopathy       | 50         | 50            | 100        | 0             | 100        | 0             |
| 20              | Endocrinopathy        | 60         | 40            | 70         | 30            | 80         | 20            |
| 6               | Hepatitis             | 85         | 15            | 75         | 25            | 100        | 0             |
| 4               | Postcholecystectomy   | 50         | 50            | 100        | 0             | 100        | 0             |
| 5               | Postcholecystostomy   | 60         | 40            | 100        | 0             | 100        | 0             |
| 1               | Avitaminosis "A"      | 100        | 0             | 100        | 0             | —          | —             |

#### MISCELLANEOUS CLINICAL FINDINGS

In chronic cholecystitis (proved by cholecystography, duodenal intubation, and surgery) complete relief of symptoms with Sorparin was obtained in 36 per cent, partial relief in 36 per cent, and no relief in 28 per cent of the cases receiving 18 grains daily. When these patients were given unconjugated-ketocholic acids (Ketocho), 7.5 to 22.5 grains daily, or iron bile salts (Bilron), 5 to 15 grains daily, the incidence of relief from symptoms was approximately the same. In some instances Sorparin gave relief when Ketocho and Bilron did not, and in some patients the reverse was true. When given alone, without antispasmodics or special diets, Sorparin produced satisfactory results in half of the patients who obtained relief. However, its action was enhanced when the diet was regulated,<sup>4</sup> and antispasmodics and sedatives were administered. Patients who had been subjected to cholecystectomy or cholecystostomy, and who had recurrence of their symptoms, obtained partial or complete relief in a slightly lower (10 to 20 per cent) percentage of instances than when no surgery was done. Sorparin will give relief in cholecystopathic dyskinesia, but it is not as effective as bile salt and/or bile acid preparations. It is an interesting fact that patients in menopause experience considerable relief when Sorparin or bile acids or bile salts are given for their associated biliary complaints, an observation in this series of cases for which an explanation is not forthcoming at this time.

The length of time Sorparin must be administered before symptomatic relief is obtained is variable, but usually results are noticeable in seven to fourteen days. But as in any other type of medical therapy no permanent cures can

be obtained. These patients need controlled supervision, re-education as to diet and habits, and correction of infections, metabolic dyeracias, and the like, which are so common in patients suffering from biliary disease. In short, Sorparin, like bile salt and bile acid preparations, is not a panacea for chronic gall bladder and liver disease, but it does dispel the symptoms in many patients who suffer from these diseases.

No toxic symptoms attributable to any of the drugs discussed in this paper were encountered.

#### COMMENTS

The extensive literature in the field relating to prothrombin and vitamin K has been reviewed by Smith and co-workers<sup>7</sup> and Warner.<sup>10</sup> That vitamin K is necessary for the production of prothrombin has been definitely established. Warner<sup>10</sup> cites as natural sources of this vitamin, green vegetables, such as alfalfa, spinach, and kale, and putrified fish meal. There have been no reports of its occurrence in fruits. McKee and his associates<sup>5</sup> found that the active principles, vitamins K<sub>1</sub> and K<sub>2</sub>, isolated from alfalfa and putrified sardine meal, respectively, have a quinoid structure. Of the numerous quinoid compounds which have been synthesized, the most potent biologically has been found to be 2-methyl-1,4-naphthoquinone.<sup>10</sup> This compound was employed in this study for comparative purposes.

Little is yet known concerning the optimum dosage of vegetable and animal concentrates or synthetic compounds of known vitamin K activity to be employed clinically. In the cases reported here 1 Gm. of the alcohol extract (Sorparin), equivalent to 2.5 Gm. of the dried berry of the European mountain ash, was the maximum daily dosage. Similar studies with extracts from other natural sources in their effects on the prothrombin level in man have been made by others. Smith and co-workers<sup>7</sup> administered extracts, representing 300 to 400 Gm. of alfalfa meal daily, and obtained a rapid rise (three to eight days) in the prothrombin level. Stewart<sup>8</sup> gave his patients a daily dosage of extracts equivalent to from 140 to 700 Gm. of spinach.

In the hemorrhagic chicks comparable results in the lowering of the clotting time were obtained by feeding one part by weight of 2-methyl-1,4-naphthoquinone to 400,000 parts of Sorparin. In respect to the prothrombin level in man the ratio by weight was one part of the pure synthetic compound to 400 parts of Sorparin. This variance in activity in the human being and in the chick is significant.

In another section the approximate analyses of the extract of the European mountain ash berry was presented. Among the known constituents of this extract are rare carbohydrates and dibasic acids and their derivatives. Information is limited concerning the degree and mode of bacterial synthesis from other materials of vitamin K in the intestinal flora of mammals. Accordingly, until adequate chemical identification and biological assay of the active principle or principles of this extract are completed, no definite reference of its activity in coagulation can be made to the presence of Vitamin K compounds with or without a quinoid configuration.

Aside from raising the plasma prothrombin level, Sorparin produced symptomatic relief of subjective complaints in a large percentage of patients (up



to 72 per cent). This observation is interesting in that Sorparin has no known chologogic or choleretic activity. The exact mode of action is not clear, since the known vitamin K compounds do not produce similar results.

### CONCLUSIONS

1. The administration of the dried berry of the European mountain ash, *Sorbus aucuparia* L., and its extracts to hemorrhagic chicks significantly reduced their clotting time. A synthetic vitamin K compound, 2-methyl-1,4-naphthoquinone, was employed as control.

2. Fourteen patients with various types of biliary and hepatic disease were investigated for their plasma prothrombin response to the alcohol extract (Sorparin). In the total of 25 periods of medication in these patients, of this extract, with or without bile acids and salts, 72 per cent showed marked increase in the prothrombin level; 20 per cent, slight increase; and 8 per cent, decrease. Significant elevation of the plasma prothrombin was observed after Sorparin therapy in all 7 cases of cholecystitis, with or without cholelithiasis. A case of idiopathic hypoprothrombinemia is reported.

3. Clinical studies reveal that approximately 72 per cent of the patients in this series afflicted with chronic cholecystitis, with or without stone, post-surgical biliary disease, and hepatitis obtained symptomatic relief when given 18.0 grains of Sorparin daily. This compares favorably with the results obtained with bile acid/salt preparations, such as Ketochole and Bilon.

We are indebted to the McNeil Laboratories for the extract of the European mountain ash berry (Sorparin); to G. D. Searle & Co. for the unconjugated-ketocholanic acids (Ketochole); to Eli Lilly and Company for the iron bile salts (Bilon).

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## LESIONS IN THE SUPERIOR MEDIASTINUM WHICH INTERFERE WITH VENOUS CIRCULATION\*

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THE superior mediastinum is the great transportation center of the body. Through it passes all food on its way to the gastrointestinal tract, all air which enters and leaves the lungs, all lymph in the thoracic ducts, all blood which leaves the heart and which returns to it from the superior half of the body. It is surprising that obstructive symptoms do not develop more frequently than they do in this crowded region. It is more surprising still when the frequency with which the numerous mediastinal lymph nodes are involved in tumefactive processes due to inflammatory, tuberculous, and neoplastic lesions is recalled.

The anterior and posterior boundaries of the superior mediastinum are firm and unyielding parts of the thoracic wall. The lateral boundaries are elastic, being separated from the spongy lungs by nothing more than the pliable pleural layers. Space-occupying lesions can thus expand laterally.

The trachea is well protected by its cartilaginous rings. Although frequently displaced, it is rarely collapsed by extrinsic pressure sufficient to produce obstructive symptoms. The esophagus lies in a somewhat protected region, and its elasticity and power of independent motility make serious functional impairment from extrinsic pressure rather uncommon. The great arterial trunks not only have firm walls, but the contained blood is under pressure sufficient to prevent interruption of the flow by any external force of reasonable intensity.

The large mediastinal veins, on the other hand, are thin-walled, and the blood flowing through them is under very low pressure. Furthermore, these veins are anteriorly situated nearer the unyielding bony thoracic cage, against which they may be compressed by expanding mediastinal lesions. These veins drain blood from the upper half of the body only: from the upper extremities, the head, neck, and thoracic wall. Obstruction to flow of blood in these important channels will produce localized symptoms and signs pathognomonic of a mediastinal lesion. Since these vessels are readily compressed, such manifestations may be early indications (sometimes even initial symptoms) of serious mediastinal disease. These phenomena may be ascribed to delayed circulation time, increased venous pressure, and collateral diversion of the blood stream. All these factors are amenable to objective study and graphic demonstration. They are not uncommon, but they are frequently overlooked and are not adequately stressed in medical literature.

\*From the Division of Medicine, the Mayo Clinic, Rochester.  
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TABLE I  
MEASUREMENT OF DIRECT VENOUS PRESSURE AND CIRCULATION TIME (DECIOLIN METHOD) IN REPRESENTATIVE CASES FROM 31 CASES OF SUPERIOR MEDIASTINAL OBSTRUCTION

| CASE | DIRECT VENOUS PRESSURE, MML. WATER |          |                            |               | CIRCULATION TIME, SECONDS |                     | PATHOLOGIC CONDITION PRESENT |
|------|------------------------------------|----------|----------------------------|---------------|---------------------------|---------------------|------------------------------|
|      | RIGHT ARM                          | LEFT ARM | LEFT EXTERNAL JUGULAR VEIN | ABNORMAL WALL | FOOT                      | RIGHT ARM TO TONGUE | LEFT ARM TO TONGUE           |
| 1    | 296*                               | 270*     |                            |               | 45                        | 70                  | 36                           |
| 6    | 292†                               | 252†     |                            |               |                           |                     |                              |
| 7    | 218†                               | 220†     |                            |               | 48                        |                     |                              |
| 9    | 232                                | 238      |                            |               |                           | 27                  | 45                           |
|      | 102                                | 100      |                            |               |                           |                     |                              |
|      | 76                                 | 172      |                            |               |                           | 29                  |                              |
| 10   | 384                                |          |                            |               |                           | 14                  |                              |
| 11   | 292                                | 299      |                            |               |                           |                     |                              |
| 12   | 356                                | 356      |                            |               |                           |                     |                              |
| 13   | 262                                |          | 286                        | 338           |                           | 28                  |                              |
| 19   | 394                                | 394      |                            |               | 48                        |                     |                              |
| 22   | 76                                 | 186      |                            |               |                           |                     |                              |
| 23   | 382                                | 416      |                            |               |                           |                     |                              |
| 25   |                                    | 294      |                            |               |                           | 20                  |                              |
| 28   | 300                                |          |                            |               |                           |                     | 20                           |
| 29   | 335                                | 345      |                            |               |                           | 29                  |                              |
| 30   | 336                                | 330      |                            |               |                           |                     |                              |
| 31   | 300                                | 290      |                            |               |                           | 54                  | 42                           |

\*Before roentgenologic treatment, June 29, 1938.

†After roentgenologic treatment, July 11, 1938.

‡After roentgenologic treatment, August 15, 1938.

This paper is based on a study of 31 cases of obstruction to the large thoracic veins. Condensed summaries of pertinent data in a few cases will be found in Tables I and II. We shall record and illustrate certain clinical aspects of mediastinal venous obstruction, with objective measurements of the circulatory phenomena demonstrated. We have seen all the patients in consultation. No effort has been made to study cases in the clinic files in which the patients were examined by other physicians.

TABLE II  
CAUSES OF MEDIASTINAL OBSTRUCTION IN 31 CASES

| CLINICAL DIAGNOSIS                  | MALES | FEMALES | TOTAL |
|-------------------------------------|-------|---------|-------|
| Carcinoma of bronchus               | 8     | 0       | 8     |
| Lymphoblastoma                      | 4     | 2       | 6     |
| "Mediastinal fibrosis"              | 5     | 3       | 8     |
| Pericardial constriction (?)        | 0     | 2       | 2     |
| Metastatic carcinoma                | 2     | 1       | 3     |
| Aortic aneurysm                     | 2     | 0       | 2     |
| Primary malignant mediastinal tumor | 1     | 1       | 2     |
| Total                               | 22    | 9       | 31    |

#### METHOD

The venous pressure was measured by the direct method. For this purpose a point of reference was taken 5 cm. below the fourth interspace at the right border of the sternum. The vein was placed at this level by means of a T-square arrangement with a carpenter's spirit level. A three-way stopcock was used between the syringe and the needle. A manometer tube graduated in millimeters was attached to the upright portion of the stopcock, and the direction of flow was controlled by means of a three-way valve. A 3 per cent solution of sodium citrate was placed in the syringe to prevent clotting of the blood. Venipuncture was made in the ordinary manner, after which the mixture of blood and citrate was forced up into the manometer tube. When the manometer tube was connected directly with the vein, the fluid fell in the manometer tube until its pressure equaled that in the veins. By this method the normal venous pressure was found to vary from 40 to 120 mm. of water. Clinically, it was found that the pressure in the veins could be simply estimated by observation of the level above the heart at which the veins on the dorsum of the hand would collapse when the arm was raised.

The circulation time was measured by injection of 5 c.c. of a 20 per cent solution of sodium dehydrocholate\* into the antecubital vein; the time that elapsed before a bitter taste was noted in the patient's mouth was checked by a stop watch. This procedure constituted rough measurement of the pulmonary circulation time; normally, it varies from twelve to seventeen seconds.

Infrared photographs were made by the Mayo Clinic Department of Photography under the direction of Mr. Leonard A. Julin. Films sensitive to infrared rays and a suitable filter were used.

#### SYMPTOMS OF MEDIASTINAL OBSTRUCTION

The symptoms commonly produced by compression of the great veins of the thorax are characteristic. In most of our cases the lesion had developed

\*Decholin (Riedel-DeHaen, Inc., New York N. Y.)

rather slowly and had been present long enough to permit fair circulatory compensation to develop. If obstruction develops more rapidly, alarming edema of the upper half of the body may occur.

Most patients in our series were relatively comfortable as long as gravity was aiding the return flow of blood. Distress was manifest chiefly on stooping or bending forward, or when exertion accelerated the flow of blood beyond the capacity of the anastomotic vessels. Undoubtedly, temporary increased cerebral congestion occurred at such times to explain the vertigo, throbbing sensations, and sometimes confusion and headache of which the patients complained. In one instance, syncope was frequent, and in another instance, convulsions occurred. Externally, suffusion and cyanosis of the face were seen, with very prominent engorgement of the veins of the forehead, face, and neck, and sometimes apparent prominence of the eyes. On the patient's resuming erect posture,



Fig. 1.—*a*, Infrared photograph of thorax; superior vena caval obstruction; note anastomoses between jugular veins, cephalic veins, and perforating branches of the internal mammary veins. *b* (Case 2), Mediastinal fibrosis; infrared photograph showing anastomotic veins on anterior thoracic wall caused by superior vena caval obstruction.

many seconds might pass before circulatory conditions became stabilized. Although moderate orthopnea frequently was mentioned, the symptoms were minimal at night, when the circulatory rate was slowed naturally because of inactivity. Dyspnea was a frequent complaint and was thought to be caused more by circulatory embarrassment than by obstruction of the air passages.

#### LOCALIZATION OF THE OBSTRUCTING LESION

Blood from the upper extremities drains into the subclavian veins. These are joined by the jugular veins to form the innominate veins. The right and left innominate veins unite to form the superior vena cava. Near the upper end of the superior vena cava the azygos vein contributes blood from the thoracic wall. The superior vena cava ends in the right auricle.

If the superior vena cava is obstructed above the level of the azygos vein, the latter may serve as an important collateral channel. In this instance, blood from the head and neck will follow the external jugular veins to a plexus on the anterior thoracic wall. Blood from the arms will reach the same plexus by way of the cephalic veins. This plexus may form prominent anastomoses with the perforating branches of the internal mammary veins, and thence to the intercostal veins which are drained into the azygous system (Fig. 1*a* and *b*).

If surgical treatment is being considered, it is desirable to localize the site of obstruction accurately. In other cases it is interesting to do so, and prognosis may be affected by the findings.

Simple inspection and the determination of direct venous pressures should be adequate to localize obstruction of high degree. If a subclavian vein is involved, the phenomena of distention and increased pressure will be limited to the veins of one arm. If the veins of one side of the neck, especially the external jugular vein, and the veins of the arm on the same side also are involved, the innominate vein on that side also will be involved (Fig. 2a). The left innominate vein is much more frequently involved because of its longer course.

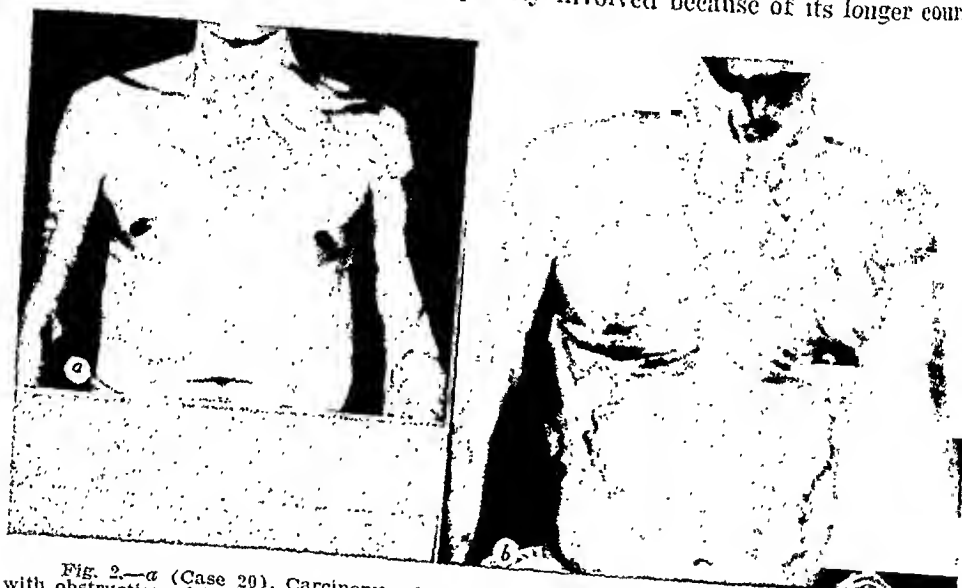


Fig. 2.—a (Case 20), Carcinoma of the bronchus, left upper lobe of lung and metastasis with obstruction of the left innominate vein; note asymmetric distribution of anastomatic veins. b (Case 18), Mediastinal lymphoblastoma; the superior vena cava is obstructed below the level of the azygos vein; note lateral abdominal veins.

If the veins of the neck on both sides and in both arms are similarly involved, the lesion may be considered to be situated in the superior vena cava. We believe that it is possible to determine further whether the obstruction is situated above or below the level of the azygos vein in certain instances of obstruction of high degree. A prominent plexus of large veins may be seen over the sternum, often clearly connecting with perforating branches of the internal mammary vein. It becomes obvious that blood is seeking a route to the intercostal vessels which drain into the azygous system. It, therefore, may be concluded that the obstruction is situated above the level of the azygos vein. Because of the relative efficiency of this route of communication, in these cases venous pressures are not so high, symptoms are not so severe, and external appearances are most striking.

If the entire superior vena caval system, including the azygos tributaries, is obstructed, blood must return to the heart through the inferior vena cava. Large veins in such circumstances cross the costal margin, and blood flows downward over the abdominal wall (Figs. 2b, 3a and b and 4). Venous pressures are high and symptoms are severe. If obstruction is situated in the superior

vena cava below the entrance of the azygos vein, establishment of effective collateral channels becomes much more difficult. It then becomes necessary for blood to seek a route to the inferior caval system. The resultant collateral channels resemble those which are formed in portal and inferior vena caval



Fig. 3 (Case 1).—*a*, Mediastinal sarcoma; the superior vena cava is obstructed below the level of the azygos vein; note the anastomotic veins on the abdominal wall. *b*, Roentgenogram of the thorax of the same patient.



Fig. 4.—Specimen of superior vena cava obtained at necropsy in Case 1. The photograph has been retouched. Extrinsic pressure had compressed the vein, producing intimal adhesions which partially blocked the channel. Point *a* was adherent to *a*, leaving vessel open only from point *a* to point *b*.

obstruction, except that blood flows in the opposite direction. Prominent tortuous vessels cross the costal margin and pass downward, chiefly in the lateral epigastric region on either side. These vessels may be traced toward the groin. It is possible to determine the direction of flow by compression of both ends of a segment of the vein with the fingers after the blood has been pressed out. If the lower finger is released, the vein refills slowly or not at all, whereas if the upper finger is removed, the vein refills almost instantly.



Fig. 5 (Case 4).—Infrared photograph of left side of thorax: carcinoma of bronchus with superior vena caval obstruction. Note dilated veins and fine superficial varicosities in the inferior midaxillary region.

#### COMMENT

There is another type of visible vascular change that is produced by mediastinal lesions. It is unlike the large, tortuous varicosities previously described and illustrated. It consists of small, superficial, rather tortuous groups of dilated, purplish venules, closely grouped in bunches and invariably situated along the anterior costal margins. The blood contained within these venules appears to be under increased pressure. It is difficult to photograph these vessels except with the aid of color films, but they are shown fairly well in Fig. 5 in contrast to the large veins. They are somewhat similar to the fine venules situated across the costal margins of normal persons, especially elderly men, and those who have a thorax of the shape of a thorax affected by emphysema. When a mediastinal tumor is present, these venules have a much more engorged appearance, and the vessels are larger, more numerous and more tortuous. They have a dark purplish appearance, unlike the fine pink or light blue hairline vessels situated along the costal margins of apparently normal persons. When the venules are associated with progressive mediastinal lesions, they usually appear rather suddenly, attracting the patient's attention. We have seen a few instances in which these small varicosities in the costal margins constituted the first objective evidence of mediastinal disease, and their significance was not realized by either patient or physician. We believe that the



rapid development of such vessels in this situation constitutes definite indication for roentgenographic study of the structures within the thorax.

Circulatory disturbances were the most prominent clinical features in many cases in this series. In more than a third (12) of the cases the chief disturbance which persuaded the patient to seek medical aid was of circulatory origin. In about a fourth (9) of the cases there was definite circulatory embarrassment, but this condition was secondary in importance to others. In the remaining cases minimal or no subjective discomfort was reported, despite objective evidence of impaired circulation.

It is important to note that the circulatory phenomena may be the earliest manifestations of mediastinal disease. They were noted as prominent early symptoms in at least half (16) of the cases of this series. In several instances experienced physicians had failed to recognize these symptoms as early evidences of mediastinal obstruction. In other instances it was possible to predict that malignant disease had already metastasized to mediastinal lymph nodes because of the circulatory disturbances noted. This conclusion afforded valuable evidence of inoperability in cases of malignant thoracic disease.

The duration of symptoms previous to diagnosis naturally depended somewhat on the nature of the obstructing lesion. In cases in which lesions were caused by malignant tumors, symptoms were of recent origin and of progressive severity. In cases of "mediastinal fibrosis" symptoms often had been present for years without evidence of progression. In 5 cases symptoms had been present for only a few weeks; in 8 additional cases symptoms were first noted within six months of the time we examined the patients. Eight other patients had noted circulatory disturbances for periods of from one to eight years. Ten patients had minimal symptoms or none at all.

Although prolonged duration of symptoms is convincing evidence of a benign lesion, a history of recent progressive symptoms does not exclude the presence of a benign inflammatory lesion. When symptoms were of recent origin, progressive and severe, with tumor not demonstrable in roentgenograms, we were inclined to regard the lesion as being inflammatory, and in three such cases, in which the patients underwent surgical exploration, such a conclusion proved to be correct.

The objective circulatory measurements recorded appear in Table I. Photographs made by infrared radiation show a striking exaggeration of the visible venous collateral channels. In no instance did infrared photography establish a diagnosis which could not have been made by easier methods. The value of this procedure is apparently limited to the production of illustrations for studies such as this one.

The clinical diagnoses and distribution of cases according to sex of patient are summarized in Table II. The great preponderance of men is due in part to the well-known fact that carcinoma of the bronchus is encountered much more frequently among men than among women.

The term "mediastinal fibrosis," as we have employed it, is not entirely satisfactory and deserves explanation. We have used it to denote cases in which tumor could not be demonstrated roentgenographically and in which duration of symptoms or surgical findings appeared to exclude malignant disease. In 3 cases in which the patients underwent surgical exploration the obstruction ap-

peared to result from fibrosis, and the fibrotic process probably surrounded healing tuberculous lymph nodes of the mediastinum. In 2 other cases roentgenographic evidence of unusually prominent calcified lymph nodes in the superior mediastinum was noted. In the remaining 3 cases long-standing symptoms of obstruction for periods of years made this clinical diagnosis appear probable. Measurements of circulatory changes were obtained in 6 of the 8 cases (Table I).

In the 2 cases in which the condition was recorded as being caused by pericardial constriction, such constriction was not proved to exist. Evidence indicated the obstruction to be situated below the entrance of the azygos vein into the superior vena cava, and in both cases severe rheumatic heart disease was present. In the remaining cases the diagnosis was based on results of bronchoscopy, biopsy, necropsy, or roentgenography.

The therapeutic test of roentgenologic irradiation frequently was employed when types of lymphoblastoma were suspected to be present. It was realized that such treatment might increase the harmful effects of mediastinal fibrosis if undertaken too vigorously; hence, the treatment usually was restricted to dosage considered adequate to accomplish the therapeutic test.

#### SUMMARY AND CONCLUSIONS

Simple inspection is adequate to reveal evidence of serious venous blockage in the superior mediastinum and to indicate the probable site of the lesion. Infrared photography will produce a striking record for illustrative purposes, but it is not necessary for diagnostic purposes. The increase in venous pressure in the arms can be roughly shown by determination of the level at which the veins empty on elevation of the arms. Direct measurement of venous pressure affords a much more nearly accurate record. Accurate measurements of venous pressure have practical value when it is desired to know whether a lesion is progressing or regressing, especially following irradiation therapy, or when surgical intervention is contemplated. The circulation time may be greatly prolonged beyond the point of obstruction in the vein.

The prominence of collateral veins bears no close relationship to severity of symptoms. As collateral varicosities increase in size, symptoms may regress. The height of venous pressure is not so closely correlated with symptoms as might be expected. Symptoms are more severe if the superior vena cava is obstructed below the point at which the azygos vein enters it, although visible varicosities may be less striking. When obstruction develops above the level of the azygos vein, symptoms are milder and collateral channels are more impressive than they are when obstruction occurs below the level of the azygos vein.

Prognosis is dependent strictly on the nature of the lesion. If it is not progressive, a degree of compensation is likely to develop; this may permit the patient to live indefinitely in fair comfort.

# CLINICAL CHEMISTRY

## ANDROGENIC AND ESTROGENIC SUBSTANCES IN URINE OF EUNUCHOID AND CASTRATE MEN\*

### CHANGES FOLLOWING ADMINISTRATION OF TESTOSTERONE PROPIONATE

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#### METHODS, MATERIALS, AND SUBJECTS

THE present use of castration as a therapeutic procedure in men with metastatic prostatic carcinoma (Huggins and associates, 1941) has led us to summarize our studies and to review the literature pertaining to biological assay of androgens and estrogens in castrate and eunuchoid men and to indicate changes in these values found upon administration of exogenous androgens.

All samples of urine were collected over periods of twenty-four consecutive hours, usually four or more days being included in each specimen. The exact number of days is listed for each subject in Table I. Urine was preserved with toluene and extracted within two weeks, except that of Case 7, the extraction of which was delayed for four weeks. Extraction was done with benzene in a continuous extractor by the method of Gallagher and others (1937). A minimum of 15, and usually 20, chicks were used for each androgenic assay which was made according to the method of Dorfman and Greulich (1937). Determinations are held to be accurate within  $\pm 25$  per cent but their accuracy is probably greater. Values are expressed in international units (I.U.), one I.U. being equivalent to the increase in comb weight evoked by 0.1 mg. of androsterone.

After extraction of the urine with benzene in a continuous extractor, the alkali-soluble fraction of the urinary extract was separated and assayed for estrogenic activity in adult spayed mice. Ten or more mice were used for each estrogenic assay. The results were expressed in international units (I.U.), one I.U. being equal to the estrous growth of the vaginal epithelium evoked by 0.1  $\mu$ g of estrone. The accuracy of the method is probably at least  $\pm 50$  per cent.

The subjects were 4 castrated men, 2 oöphorectomized women, and 7 hypogonadal men. Five hypogonadal men are prepubertal eunuchoids; two (Cases 1 and 6) were deprived postpubertally of adequate testicular function in attempted surgical repair of hernia. The term *eunuchoid* is applied to men who, although possessing testes, do not have adequate testicular function. Brief description has been given of Case 4 (Hamilton, 1937). For each patient the age and number of years since castration are listed in Table I.

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TABLE I  
VALUES OBTAINED BY VARIOUS INVESTIGATORS FOR URINARY ANDROGENS AND ESTROGENS IN EUNUCHOID AND CASTRATE MEN

| AUTHOR AND CASES         | AGE  | YEARS SINCE CASTRATION | ANDROGENIC ACTIVITY (I.U.) | PERIOD TESTED | METHOD OF ASSAY        | ESTROGENIC ACTIVITY (I.U.) | PERIOD TESTED | METHOD OF ASSAY             | RATIO OF ANDROGENS TO ESTROGENS |
|--------------------------|------|------------------------|----------------------------|---------------|------------------------|----------------------------|---------------|-----------------------------|---------------------------------|
| CASTRATE MEN             |      |                        |                            |               |                        |                            |               |                             |                                 |
| Kenyon and others (1937) | 21   | 2-3                    | *1.66 (liter)              | 18 days       | Capon comb (Injection) | 30 (liter)                 | 18 days       | Spayed adult female rats    | 0.5                             |
| R. S.                    | 56   | 36                     | *5.8 (liter)               | 20.2 liters   | Capon comb (Inunction) | 45 (liter)                 | 20.2 liters   | Spayed female rats and mice | 0.13                            |
| J. W.                    |      |                        |                            |               |                        |                            |               |                             |                                 |
| Hansen† (1938)           |      |                        |                            |               |                        |                            |               |                             |                                 |
| U1                       | 37.5 | 2                      | >15 (liter)                |               |                        |                            |               |                             |                                 |
| U2                       | 57.5 | 7                      | >2.9 (liter)               |               |                        |                            |               |                             |                                 |
| U3                       | 47   | 1                      | 15.4 (liter)               |               |                        |                            |               |                             |                                 |
| U4                       | 62   | 6.5                    | 4.7 (liter)                |               |                        |                            |               |                             |                                 |
| U6                       | 35.5 | 4.5                    | >10 (liter)                |               |                        |                            |               |                             |                                 |
| U12                      | 48   | 5                      | 2.6 (liter)                |               |                        |                            |               |                             |                                 |
| E4                       | 27   | 0.5                    | 21.8 (liter)               |               |                        |                            |               |                             |                                 |
| E5                       | 41   | 2.5                    | 4.1 (liter)                |               |                        |                            |               |                             |                                 |
| E7                       | 29   | 0.17                   | 8 (liter)                  |               |                        |                            |               |                             |                                 |
|                          |      |                        | 3.4 (liter)                |               |                        |                            |               |                             |                                 |
|                          |      |                        | 6.9 (liter)                |               |                        |                            |               |                             |                                 |
|                          |      |                        | <7 (liter)                 |               |                        |                            |               |                             |                                 |
| E10                      | 26   | 0.5                    | 5.7 (liter)                |               |                        |                            |               |                             |                                 |
|                          |      | 0.08                   | 5.1 (liter)                |               |                        |                            |               |                             |                                 |
| V11                      | 38   | 0.04                   | <5 (liter)                 |               |                        |                            |               |                             |                                 |
|                          |      |                        | >2.7 (liter)               |               |                        |                            |               |                             |                                 |
| V13                      | 29   | 0.5                    | <5.5 (liter)               |               |                        |                            |               |                             |                                 |
|                          |      |                        | >8.8 (liter)               |               |                        |                            |               |                             |                                 |
|                          |      |                        | 17.5 (liter)               |               |                        |                            |               |                             |                                 |
| McCullagh (1939)         | 64   |                        | 0,0,0,0 (day)              |               | (Intramuscular)        |                            |               |                             |                                 |
|                          | 52   |                        | 7 (day)                    |               |                        |                            |               |                             |                                 |
|                          | 42   |                        | 16,0,10 (day)              |               |                        |                            |               |                             |                                 |

The techniques of assay and extraction are listed in instances where the data are available. Only values convertible to I.U. are used. Apparently eunuchoid men usually have higher levels, and a range of values is observed in normal men. Values for urinary estrogens are also lower than those observed in normal men.

\*Values corrected as of a fifteen minute period of boiling.

†Extracted by boiling with solvent; continuous extraction used by all other authors.

| Callow and others<br>(1940) |    |    | ca. 28<br>(liter)<br>(day) | (Intramuscular) | ca. 6 (liter)<br>(day) |        | Spayed adult<br>female mice |      |
|-----------------------------|----|----|----------------------------|-----------------|------------------------|--------|-----------------------------|------|
|                             |    |    |                            |                 |                        |        |                             |      |
| L.                          | 24 | 1  | 11 (day)                   |                 | <2 (day)               |        |                             |      |
| A. R.                       | 46 | 22 | 11 (day)                   |                 | <4 (day)               |        |                             |      |
| G. B.                       | 45 | 21 | 11 (day)                   |                 | 6 (day)                |        |                             |      |
| F. F.                       | 45 | 20 | 7 (day)                    |                 | <1.5 (day)             |        |                             |      |
| F. C.                       | 45 | 10 | 10 (day)                   |                 | 12.5 (day)             |        |                             |      |
| E. C.                       | 19 | 5  | 18 (day)                   |                 | 8 (day)                |        |                             |      |
| D. S.                       | 38 | 19 | 21 (day)                   |                 | 6 (day)                |        |                             |      |
|                             |    |    |                            |                 |                        |        |                             |      |
| Hamilton, Dorf-             |    |    |                            |                 |                        |        |                             |      |
| man and Hubert              |    |    |                            |                 |                        |        |                             |      |
| Case 8                      | 45 | 26 | 3 (day)                    | Chick comb      | 11 (day)               | 4 days |                             | 0.27 |
| Case 9                      | 43 | 22 | 8 (day)                    | (Subcutaneous)  | 28 (day)               | 1 day  |                             | 0.28 |
| Case 10                     | 56 | 13 | 9 (day)                    |                 | 5 (day)                | 6 days |                             | 1.8  |
| Case 11                     | 39 | 13 | 11 (day)                   |                 | 15 (day)               | 4 days |                             | 0.73 |

## EUNUCHOID MEN

| Kenyon and others<br>(1937) |    |  | ca. 28.3<br>(liter)<br>(day) | 6.5 days<br>(day) | Capon comb<br>(Intramuscular) | 20 (liter)<br>(day) | 6.5 liters<br>(day) | Spayed adult<br>female rats |      |
|-----------------------------|----|--|------------------------------|-------------------|-------------------------------|---------------------|---------------------|-----------------------------|------|
|                             |    |  |                              |                   |                               |                     |                     |                             |      |
| N. T.                       | 26 |  | *28.3 (day)                  | 7 days            |                               | 20 (day)            | 7 days              |                             | 1.41 |
|                             | 27 |  | 0 (day)                      | 7 days            |                               | 20 (day)            | 7 days              |                             | 9    |
|                             | 27 |  | *15 (day)                    | 10 days           |                               | 20 (day)            | 10 days             |                             | 0.75 |
|                             | 29 |  | 15 (day)                     | 7 days            |                               | 10 (day)            | 7 days              |                             | 1.5  |
| F. R.                       | 24 |  | *15 (day)                    | 7 days            |                               | 20 (day)            | 7 days              |                             | 0.75 |
|                             | 24 |  | *18.3 (day)                  | 7 days            |                               | 20 (day)            | 7 days              |                             | 0.91 |
|                             | 26 |  | 28 (day)                     | 7 days            |                               | 20 (day)            | 7 days              |                             | 2.80 |
| N. D.                       | 29 |  | *18.3 (day)                  | 7 days            |                               | 10 (day)            | 7 days              |                             |      |
|                             | 29 |  | 7 (day)                      | 7 days            |                               | 10 (day)            | 7 days              |                             | 0.7  |
| H. K.                       | 23 |  | *28.3 (day)                  | 7 days            |                               | 10 (day)            | 7 days              |                             | 2.83 |
|                             | 23 |  | 18 (day)                     | 6 days            |                               | 20 (day)            | 6 days              |                             | 0.9  |
| A. M.                       | 24 |  | 12 (day)                     | 3 days            |                               | 90 (day)            | 3 days              |                             | 0.13 |
|                             | 20 |  | 21 (day)                     | 3 days            |                               | 50 (day)            | 3 days              |                             | 0.42 |
| D. V.                       | 31 |  | 21 (day)                     | 3 days            |                               | 10 (day)            | 3 days              |                             | 2.1  |
| J. H.                       | 36 |  | 33 (day)                     | 3 days            |                               |                     |                     |                             |      |
| L. H.                       | 30 |  | *6.7 (liter)                 | 11 days           |                               | 30 (liter)          | 11 liters           |                             | 0.22 |

TABLE I—CONT'D

| AUTHOR AND CASES             | AGE | YEARS SINCE CASTRATION | ANDROGENIC ACTIVITY (I.U.) | PERIOD TESTED | METHOD OF ASSAY            | ESTROGENIC ACTIVITY (I.U.) | PERIOD TESTED | METHOD OF ASSAY          | RATIO OF ANDROGENS TO ESTROGENS |
|------------------------------|-----|------------------------|----------------------------|---------------|----------------------------|----------------------------|---------------|--------------------------|---------------------------------|
| McCullagh (1939)             | 20  |                        | 9 (day)                    |               | Capon comb (Intramuscular) |                            |               |                          |                                 |
|                              | 21  |                        | 16,13 (day)                |               |                            |                            |               |                          |                                 |
|                              | 23  |                        | 2,6,5 (day)                |               |                            |                            |               |                          |                                 |
|                              | 26  |                        | 7,14 (day)                 |               |                            |                            |               |                          |                                 |
|                              | 30  |                        | 23 (day)                   |               |                            |                            |               |                          |                                 |
|                              | 31  |                        | 0,0,0 (day)                |               |                            |                            |               |                          |                                 |
|                              |     |                        | 2,1,0 (day)                |               |                            |                            |               |                          |                                 |
|                              | 33  |                        | 2,3,11 (day)               |               |                            |                            |               |                          |                                 |
|                              | 34  |                        | 7,2 (day)                  |               |                            |                            |               |                          |                                 |
|                              | 52  |                        | 2,0 (day)                  |               |                            |                            |               |                          |                                 |
| Callow and others (1940)     |     |                        | 0,1,2 (day)                |               |                            |                            |               |                          |                                 |
|                              | 42  |                        | 5 (day)                    |               |                            | ca. 12 (day)               |               |                          |                                 |
|                              |     |                        | 9 (day)                    |               |                            | 6 (day)                    |               |                          |                                 |
|                              |     |                        | 17 (day)                   |               |                            | 7 (day)                    |               |                          |                                 |
| W. H. F. D. T. C.            |     |                        | 12.5 (day)                 |               |                            | ca. 4 (day)                |               |                          |                                 |
|                              |     |                        | 20 (day)                   |               |                            | 3.5 (day)                  |               |                          |                                 |
|                              |     |                        | 14 (day)                   |               |                            |                            |               |                          |                                 |
|                              |     |                        |                            |               |                            |                            |               |                          |                                 |
| Hamilton, Dorfman and Hubert | 35  |                        | 13.5 (day)                 | 6 days        | Click comb (Subcutaneous)  | 8 (day)                    | 6 days        | Spayed adult female mice | 1.69                            |
|                              | 26  |                        | 9 (day)                    | 8 days        |                            | 31 (day)                   | 8 days        |                          | 0.3                             |
|                              | 22  |                        | 20.5 (day)                 | 4 days        |                            | 22 (day)                   | 4 days        |                          | 0.93                            |
|                              | 28  |                        | 14 (day)                   | 6 days        |                            | 12 (day)                   | 6 days        |                          | 0.17                            |
|                              | 27  |                        | 24 (day)                   | 4 days        |                            | 15 (day)                   | 4 days        |                          | 1.6                             |
|                              | 42  |                        | 23 (day)                   | 5 days        |                            | 35 (day)                   | 2 days        |                          | 0.78                            |
|                              |     |                        |                            |               |                            | 25 (day)                   | 2.5 days      |                          | 0.54                            |
|                              | 18  |                        | 27 (day)                   | 4 days        |                            | 50 (day)                   | 4 days        |                          |                                 |

Twenty milligrams of testosterone propionate\* in 1 c.c. of peanut oil were administered intramuscularly from three to seven times weekly, as described individually for each patient in Tables I and II.

## RESULTS AND DISCUSSION

*Urinary Androgens and Estrogens in the Eunuchoid and Castrate.*—(1) *Androgens.* Until recently androgens have been reported to be absent in urine of castrate men (McCullagh and Renshaw, 1934), but with the use of better methods positive findings have been recorded. Data from studies in which the excreted substances were calculated in international units of activity are abstracted and compared in Table I, together with the additional cases reported at this time.

TABLE II

URINARY LEVELS OF ANDROGENS AND ESTROGENS PER TWENTY-FOUR HOURS BEFORE AND DURING PERIODS OF DAILY INJECTION INTRAMUSCULARLY WITH 20 MG. OF TESTOSTERONE PROPIONATE IN 1 C.C. OF PEANUT OIL

| CASE | BEFORE ADMINISTRATION OF HORMONE |                         |                         | DURING ADMINISTRATION OF HORMONE |   |                         |                         |
|------|----------------------------------|-------------------------|-------------------------|----------------------------------|---|-------------------------|-------------------------|
|      | NO. OF DAYS URINE COLLECTED      | ANDROGENS (I.U./24 HR.) | ESTROGENS (I.U./24 HR.) | URINE COLLECTED                  |   | ANDROGENS (I.U./24 HR.) | ESTROGENS (I.U./24 HR.) |
|      |                                  |                         |                         | NO. OF DAYS                      | DAYS SINCE BEGINNING OF TREATMENT         |                         |                         |
| 1    | 6                                | 13.5                    | 8.5                     | 11                               | 3, 4, 5, 6, 9, 10, 12, 13, 25, 26, 27, 30 | 69                      | 45                      |
| 2    | 8                                | 9.4                     | 31                      | 11                               | 3, 4, 5, 6, 9, 10, 12, 13, 30, 31, 32     | 72                      | 100                     |
| 3    | 4                                | 20.5                    | 22                      | 11                               | 3, 4, 5, 6, 9, 10, 12, 13, 30, 31, 32     | 66                      | 78                      |
| 8*   | 4                                | 3                       | 11                      | 10                               | 4, 5, 6, 7, 9, 10, 11, 12, 54, 55         | 69.4                    | 21.5                    |
| 4    | 4                                | 14                      | 12                      | 4                                | 26, 27, 28, 29                            | 89                      | 41                      |

\*Case 8 received only six injections per week.

In our series the eunuchoids have levels of urinary androgenic activity in the lowest part of the range found in normal young men, and the values for urinary androgenic activity of every eunuchoid are greater than in any castrate except Case 2 (Fig. 1). It is noteworthy that despite these facts all persons classified as eunuchoid had organic evidence of continuously inadequate testicular secretion. For example, Case 3, who excreted 20 I.U. of androgens daily, presented the most severe state of genital underdevelopment that we have observed in an adult male. The scrotum was a small, flat band of tissue without pendulous form. The penis was 2 cm. in length, 0.5 cm. in diameter, and the prepuce was undetached from the glans penis. As estimated from roentgenographic study of the wrist the osseous age was many years less than the patient's actual age.

Although strict comparability is not to be expected among studies made in different laboratories, since the methods employed differed in several details, the various data are uniform in suggesting that the castrate rarely excretes more than 20 I.U. of androgenically active material daily, whereas the levels in the eunuchoid are usually higher than in castrates and may be within the lower limits found in normal men. The daily values in normal men have been stated by Gallagher and co-workers (1937) to be 22 to 132 I.U., by Dingemans and associates (1937), 15 to 170 I.U., and by Callow and co-workers (1938), 20 to 110 I.U.

\*Testosterone propionate was furnished through the courtesy of the Ciba Pharmaceutical Company Products, Inc., Summit, N. J., under the trade name Perandren.

(2) *Estrogens.* The presence of urinary estrogens has been demonstrated for castrate men and oöphorectomized women (Bingel, 1935; Hansen, 1936a; Kenyon and associates, 1937). In the patients reported by the present authors the range of values is 5 to 28 I.U. for castrates, 8 to 50 I.U. for eunuchoids (Table I). These titers are less than those reported for normal men whose range in daily values is 20 to 290 I.U. according to Gallagher and associates (1937).

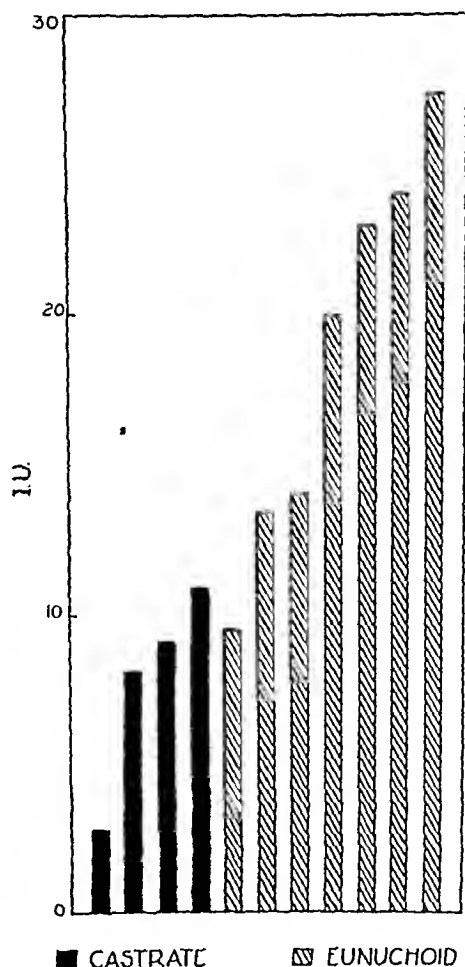


Fig. 1.—Daily excretion of urinary androgens in four castrate and eunuchoid men. All men excrete appreciable quantities of androgens, but the output of the castrate man (average 7.5 I.U.) does not fall within the range, much less the average of normal men (range from about 20 to 120 I.U., average about 60 I.U.). The range of excretion among the eunuchoid group extends from that of the castrate to the lower limits of the range in normal men. Data for men with testicular insufficiency are from cases reported by Hamilton and Dorfman in Table I.

#### COMMENT

The testes play a major role in men in the production of both androgens and estrogens. Orchidectomy or hyposecretion of the testes is accompanied by subnormal levels but not by absence of sex hormone activity in the urine. Extragonadal sources of these urinary steroids or their precursors may be the adrenal glands, as indicated by the excretion of large quantities of steroids in



certain patients with adrenal tumors (Slot, 1936; see Dorfman and co-workers, 1940), but specific designation of these glands as the sole source is not warranted by available data.

# EFFECT OF ADMINISTRATION OF ANDROGENS UPON URINARY LEVELS OF GONADAL SUBSTANCES IN THE EUNUCHOID AND CASTRATE

*Urinary Androgens.*—In 1937 Koehakian reported that after injection of male hormone extracts from urine and of androstendione, about 2 to 6 per cent of the activity represented in the administered substances could be recovered from the urine. Callow (1938) found that after injection of testosterone propionate there was very little additional or even less urinary androgenic activity save in the case of two eunuchs who received fairly large doses. In 1939 (Dorfman and Hamilton) assays of urine from eunuchoids receiving testosterone propionate showed considerable increase in androgenic titers, which in one instance in terms of molecular conversion of testosterone to androsterone, amounted to approximately 41.4 per cent. In man conversion of testosterone to androsterone has been demonstrated by the isolation of large quantities of androsterone from urine of patients receiving testosterone (Callow, 1939; Dorfman, Cook, and Hamilton, 1939).

TABLE III

PERCENTAGE OF ADMINISTERED ANDROGEN RECOVERED AS URINARY ANDROGENS AND ESTROGENS

| CASE | RECOVERY AS ANDROGENS<br>(CALCULATED AS ANDROSTERONE) |  | RECOVERY AS ESTROGENS<br>(CALCULATED AS ESTRONE) |  |
|------|---|--|--|--|
|      | TOTAL EXCRETION<br>(%)                                | WITH ALLOWANCE<br>FOR POSSIBLE ENDOG-<br>ENOUS ANDROGEN<br>(%) | TOTAL EXCRETION<br>(%)                           | WITH ALLOWANCE<br>FOR POSSIBLE ENDOG-<br>ENOUS ESTROGEN<br>(%) |
| 1    | 41.4  | 32.9   | 0.03   | 0.02   |
| 2    | 43.1  | 37.7   | 0.06   | 0.04   |
| 3    | 39.5  | 27.6   | 0.05   | 0.03   |
| 8    | 48.3  | 46.1   | 0.02   | 0.02   |
| 4    | 53.2  | 44.8   | 0.01   | 0.01   |

The above figures were computed from data in Table II; the following instance of Case 2 illustrates the method of calculation:

Daily injection of 20 mg. of testosterone propionate = 16.7 mg. of testosterone.

Average daily androgenic excretion in days tested = 72 I.U.

72 I.U., calculated as androsterone = 7.2 mg. or 41.3% recovery.

If endogenous androgen were still excreted at levels observed prior to therapy, 72 I.U. minus 9.4 I.U. = 62.6 I.U. net excretion = 37.7% recovery.

Average daily excretion of estrogen in days tested = 100 I.U.

100 I.U. calculated as estrone = 0.01 mg. = 0.06% recovery.

If endogenous estrogen were still excreted at levels observed prior to therapy, 100 I.U. minus 31 I.U. = 69 I.U. net excretion = 0.04% recovery.

Probably the chief androgenically active compound in these urines is androsterone, as computed; but the urinary estrogenic substance, although phenolic, may not be estrone. Calculated as estriol, figures for percentage recovery are increased by a multiple of 50.

The relatively high percentage recovery in Case 4 may be due to the fact that the assays were made only after therapy had been given for several weeks. The figures would be higher also in the other cases if allowance had been made for the quantities excreted after cessation of therapy.

Values for Case 1, taken from other studies (Dorfman and Hamilton, 1939), are included in Tables II and III since these data were not calculated in the form used here.

The figure for percentage recovery (calculated as androsterone) reported previously is substantiated by data from four additional cases which are summarized in Table III and confirmed by the observations made independently by Hoskins and others (1939), and by McCullagh (1939). It is interesting that the percentage recovery is highest with subcutaneously implanted pellets of

testosterone propionate (Dorfman and Hamilton, 1941), less with intramuscular injections, and apparently even lower with oral administration (Dorfman and Hamilton, 1940).

In addition to androsterone there has been recovered from the urine of eunuchs and eunuchoids given testosterone propionate, etiocholanol-3( $\alpha$ )-17-one (Callow, 1939; Dorfman, 1940). and etioallocholanol-3( $\beta$ )-17-one (iso-androsterone) (Dorfman, 1941). Another androgen of human urine, dehydro-iso-androsterone, has not been demonstrated to be a metabolite of testosterone.

From the foregoing comments it is evident that androsterone is very largely responsible for the androgenic activity of substances found in the urine after administration of testosterone to eunuchoids. The values observed are listed in Table II, from which it will be seen that in all of these instances 20 mg. of testosterone propionate injected intramuscularly seven times (six times in Case 8) weekly sufficed to maintain during the period of treatment urinary levels of androgens of approximately the average seen in normal young men. This is in agreement with the earlier report of Dorfman and Hamilton (1939), with daily intramuscular injections of 20 mg., and is in general agreement with the findings of Hoskins and co-workers (1939), with injections of 25 mg. daily.

TABLE IV

CASE 8 SHOWING EXTREME LOWERING OF LEVELS OF URINARY ANDROGENS WHEN WEEKLY INJECTIONS OF TESTOSTERONE PROPIONATE ARE 60 MG. INSTEAD OF 120 MG.

| TREATMENT WITH 120 MG. OF<br>TESTOSTERONE PROPIONATE<br>WEEKLY | DAYS SINCE BEGINNING<br>OF THERAPY | TREATMENT WITH 60 MG. OF<br>TESTOSTERONE PROPIONATE<br>WEEKLY |
|--|------------------------------------|---|
| URINARY ANDROGENS<br>(I.U./24 HR.)                             |                                    | URINARY ANDROGENS<br>(I.U./24 HR.)                            |
|  | 2                                  | 9.2   |
|  | 3                                  | 5.2   |
| 52   | 4 and 5                            |   |
|  | 4                                  | 15  |
| 47   | 6 and 7                            |   |
| 89   | 9 and 10                           |   |
| 70   | 11 and 12                          |   |
|  | 21                                 | 13  |
| 89   | 54 and 55                          |   |

If the dose is only about one-half of the amount (20 mg. of testosterone propionate daily) usually used in the treatment of eunuchs and eunuchoids of this series, that is, if injections of 20 mg. are given only three times per week, values of urinary androgenic activity were only slightly higher than observed before treatment and strikingly reduced from values observed with double the therapeutic dose (Table III). This observation requires confirmation, for only one person was studied in detail.

*Urinary Estrogens.*—Man excretes relatively large quantities of urinary estrogens, about 90 to 120 I.U. daily, in comparison with 180 to 360 I.U. excreted by women (Gallagher and co-workers, 1937). The source of these estrogens is in part the testis, as indicated by the low estrogenic values in castrated men.

Thus it was logical to ascertain whether or not there was conversion of androgens to estrogens. Steinach and associates (1936) found that the estro-

genic activity in the urine of normal and castrate male rats was greater after administration of urinary extracts and of androsterone; Steinach and Kun (1937) observed that injection of men with 1,000 mg. of testosterone propionate in doses of 50 mg., three times weekly, resulted in increased urinary estrogenic activity from values of 0 to 36 R.U. per liter found before injections to titers of 1,200 R.U. per liter of morning samples after seven weeks of injection. Hoskins and co-workers (1939), write, "These high values are especially remarkable in view of the almost casual extraction conducted by shaking the urine twice with benzene and the absence of hydrolyzing procedures usually necessary to secure maximum yields of estrogens from urine."

Koehakian (1938) detected no augmented estrogenic activity in the urine of one normal dog and of four castrate dogs which received several testosterone compounds, androstendione, and urinary androgens. Nevertheless, despite the above evidence to the contrary, it now appears certain that in man, testosterone can be converted to a substance (s) that is active estrogenically (Hoskins and co-workers, 1939; Dorfman and Hamilton, 1939; Callow and co-workers, 1939).

Table III shows the excretion of increased amounts of estrogenic substances during the period of treatment and for a short while thereafter. Amounts much larger than those in the normal young man were not encountered under the conditions employed. The values of 0.06 and 0.02 per cent from five cases, calculated on the basis of estrone, stated independently by Hoskins and others (1939), and by Dorfman and Hamilton (1939) are in agreement, and are substantiated by data from four additional cases listed in Table III.

The question arises as to the possibility of estrogenic action exerted directly by androgens or indirectly by substances converted from testosterone. Many androgenic compounds induce uterine and mammary growth, and one, trans-androstenediol, has marked estrogenic activity (Emmens and Parkes, 1938). Tenderness and swelling of the subareolar area occur in the eunuchoid receiving testosterone (Hamilton, 1937) and spontaneously in the boy at puberty (Jung and Shafton, 1937), but a variety of substances exert an effect on the mammary glands, and there is no proof as to whether or not the estrogenic action necessarily either precedes or follows conversion of androgen to estrogen.

#### SUMMARY

The levels of androgenic and estrogenic substances in the urine of 7 eunuchoid and 4 castrate men are presented in Fig. 1 and Table I. Data existent in the literature on this subject are summarized and listed in Table I.

In the present series of cases the average daily androgenic activity of the urine of eunuchoids (18.8 I.U.) was slightly less than one-third that of normal young men, and that of the castrates was still lower (7.7 I.U.). The average daily estrogenic activity of the urine was also less than in normal young men, being 27.4 I.U. in the eunuchoid, and 14.7 I.U. in the castrate.

Daily injection of 20 mg. of testosterone propionate resulted in elevation of values for urinary androgenic activity to the range in normal young men. Urinary estrogenic activity was also increased as a result of conversion of testosterone to phenolic estrogenic substances. Upon the cessation of injections of testosterone the values for urinary androgens and estrogens returned to those found before the employment of therapy.

In terms of molecular conversion to androgens and estrogens, the percentage recovery in the urine of the administered androgen is appreciable and comparable from person to person (Table III). Apparently, recovery following intramuscular injections in oil is less than that obtained upon implantation of pellets of testosterone propionate, but is in turn greater than that occurring after ingestion.

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# LABORATORY METHODS

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## GENERAL

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### HEMATOCRIT TUBE BALANCING RACK\*

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IT IS recognized that when in use, the various sedimentation instruments for determining the sedimentation rate of whole blood must be as nearly vertical as it is possible to attain in order to prevent an increased settling rate caused by slight inclination of the tube.

Most devices to aid in securing this vertical position require either a perfectly flat surface for mounting, or incorporate a system of levels which are adjusted by a system of screw legs. The former is unsatisfactory because of the difficulty in finding a perfectly level laboratory table. The latter method is relatively complicated and requires frequent adjustments if the device is moved from table to table.

The device herein described and illustrated permits the Wintrobe type of tubes to rest within fractions of a degree of the vertical position. The balance rack may be placed on any smooth top table with disregard for the level of that table, and with one simple motion of the damping stage, fixed into a true vertical position. Tubes may then be placed into or removed from the rack without disturbing the balance. This type of balancing device is satisfactory for all styles of laboratories; for example, research, arthritic clinics, sanatoriums, and ward laboratories in hospitals. We have used it with satisfaction in this hospital in connection with further research on the problem of sedimentation.

The instrument consists essentially of (1) a rack for holding the tubes (this is supported on a pivot in pendulum fashion) and (2) a damping device for stopping this rack and holding it in a fixed position.

The *rack* is constructed of three parts. The upper part (A) is composed of a lightweight material, such as aluminum, plastic, or wood. It is circular in cross section and deep enough to contain several drilled holes into which the hematocrit tubes may be lowered about 3 cm. These holes are centered by machine to give necessary accuracy (the whole rack must, of course, be accurately machined in order to maintain a vertical position) and placed in such positions as to aid balance; this is done by placing one hole in the center, then an even number of holes at the periphery. The bottom of this piece is drilled and tapped to receive the middle part.

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The middle part (*B*) of the rack consists of a threaded joint which holds the two other parts together and serves as the fulcrum for the pivot.

The lower part (*C*) of the rack simply consists of a heavy mass of iron (1 pound or so) which receives the joint at one end; it is rounded at the lower end to offer a constant surface to the damper. The rack hangs on the pivot which is mounted on a scaffold-like arm. This is mounted on the same base as the damper.



Fig. 1.—Hematocrit tube balancing rack in use.

The *damping device* consists of (1) a soft sponge rubber block mounted on a stage which in turn is attached to a strip of sheet spring metal, and (2) an off-center cam which when worked by a suitable handle raises the stage and rubber cushion against the bottom of the rack.

The rubber block must be soft in order to press equally in all directions against the rack and to prevent lifting of the rack off the pivot; it is cemented to the stage. The stage is riveted to the strip of spring. The spring is attached firmly at one end and loosely at the other to permit up and down motion; the stage moves vertically.

The cam is half rounded at opposite edges to permit smooth and steady setting against the spring plate. A handle is joined to the cam via a shaft.

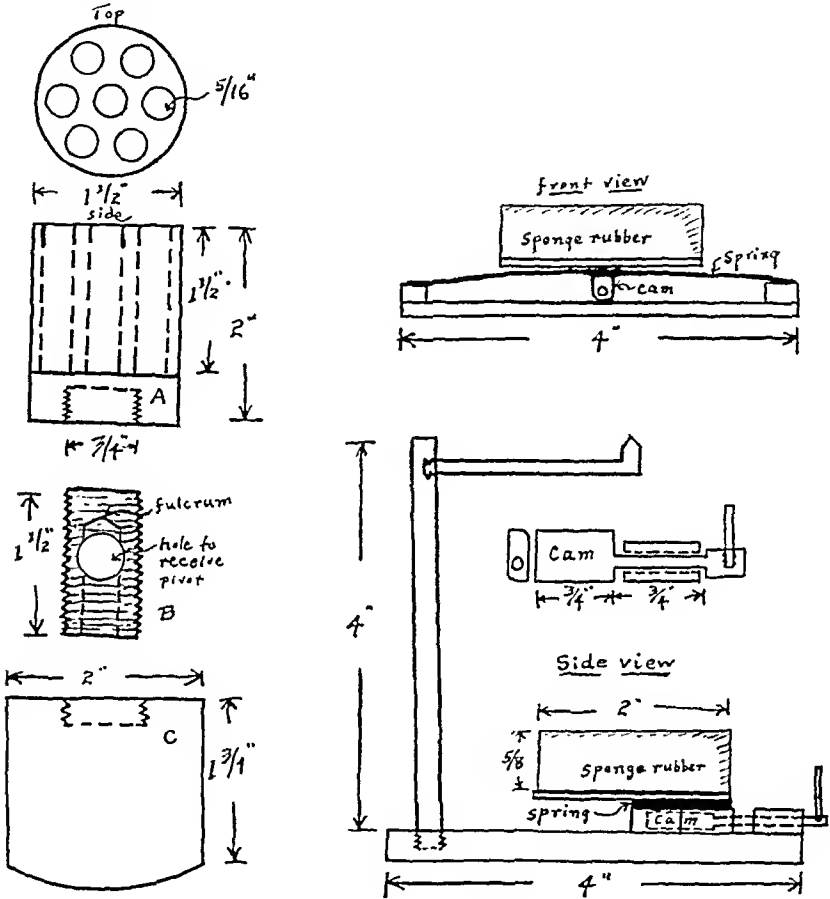


Fig. 2.—Details of construction of the rack and damping device.

When it is desired to balance one or several filled Wintrobe tubes, the tubes are placed in the rack either before or after setting; any number from one to seven (or more if more holes are drilled) may be so balanced. Being mounted on a circular rack, the tubes may be read from all sides. The rack is allowed to swing freely on the pivot, and is gradually damped down as it assumes the vertical position (regardless of the level of the base). When the vertical point is reached, the handle is snapped completely over to fix the rack in this position. There are thus no adjustments of levels to be made at any time; gravity does the balancing job, and a single turn of the thumb fixes the rack in a perfectly vertical position.



# A SIMPLE MECHANICAL FEEDING DEVICE<sup>8</sup>

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IN THE course of experiments which required that dogs be administered a drug at four-hour intervals day and night it became imperative that an automatic feeding device be employed. After several designs had been considered and rejected, the rather simple arrangement illustrated in Fig. 1 was improvised.

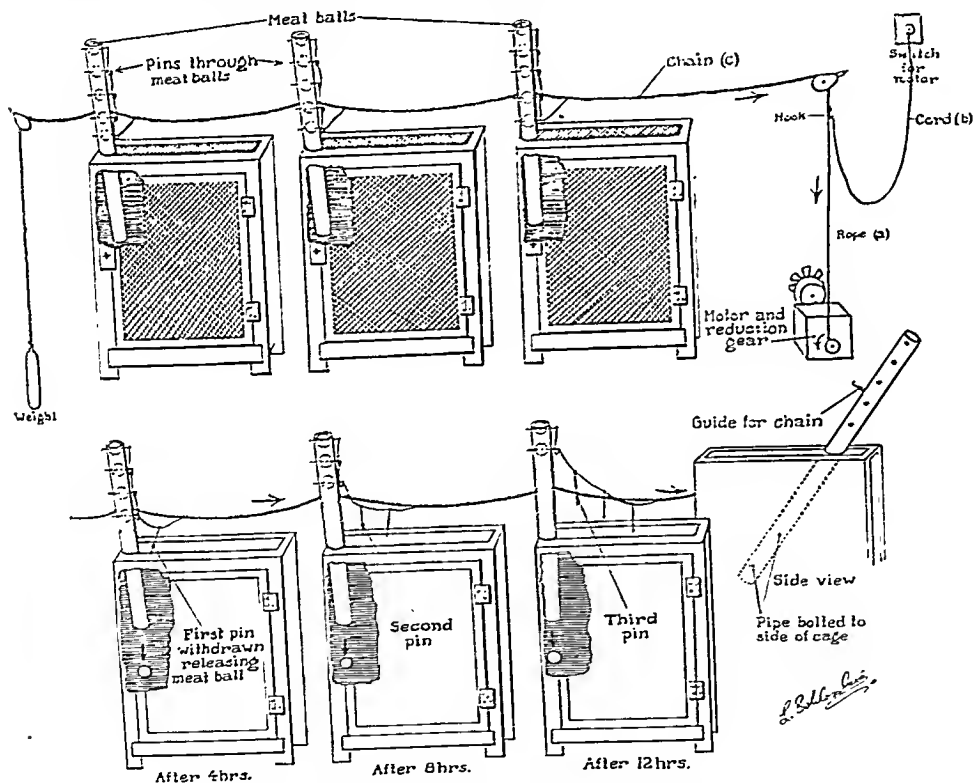


Fig. 1.—This illustration shows the position of the 2 inch iron pipe placed through the top and fastened to the side of an animal cage.

Fig. 1 requires but little explanation. A small, 1,750 r.p.m. electric motor designed for continuous operation is connected in series with two 1 to 400 reduction gears mounted in series. This arrangement will give a speed of one revolution every ninety minutes for the low-speed shaft of the second gear. On this shaft is mounted a reel onto which rope *a* will be wound to advance the chain *c*.

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The travel of this chain in a given interval of time will determine the spacing of the pins. The device as used here was designed to feed every four hours. In order that the feeder need attention only once daily, five pins were incorporated in the design. Pull cord *b* is adjusted so as to throw the motor switch after the last pin has been withdrawn. Rope *a* is attached to chain *c* by a hook to facilitate unwinding the rope at the completion of a twenty-hour run.

The daily dosage of drug was mixed with a portion of ground meat which was divided and made into six similar balls. Five of these balls are skewered with the pins and are released to fall into the animal's cage as each pin is withdrawn. An economical feeding mixture consists of ground, boiled lungs cemented together with a small amount of admixed tallow. The animal's total daily food ration can be incorporated in these meat balls. To insure the administration of the drug at the desired time the animals are kept slightly hungry and they eat the food immediately when it is delivered.

Two tiers of cages arranged on three walls of a room were operated by a single mechanism. If the animal is given a feeding when the feeder is loaded, it will demand attention once daily. With slight changes the device can be altered for feeding different types of food.

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## PREPARING PARAFFIN FOR IMBEDDING PURPOSES\*

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FRESH paraffin is unfit for microtechnical purposes, since it contains volatile admixtures which make the paraffin brittle, thus rendering sectioning difficult. Besides, fresh paraffin often crystallizes. Therefore Apathy advised that fresh paraffin be left for about one week at 70° to 80° C. and filtered several times. Spee mentioned that overheating renders paraffin fit for sectioning in ribbons. Other authors (Schulgin, Brass, Altmann, Pohlmann, van Walsen, Kabsch) advocated the admixture of wax, ceresin, myrtle wax, mastix, vaseline, etc.

The following is a procedure for rendering paraffin suitable for sectioning. It combines the advantages of overheating, as advocated by Spee, with those of the addition of wax, as suggested by Brass, van Walsen, and Kabsch, the disadvantages of the various methods being as far as possible eliminated.

The paraffin is purified through prolonged heating (see *A*) and made smooth through the addition of beeswax. The wax is heated for a short time in order to prevent the surface of the compound from becoming greasy. Thus, 10 per cent of beeswax may be added. It is an advantage that the melting point of the compound is almost the same as that of the original paraffin. If

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\*From the Zoological Institute of the Hebrew University, Jerusalem.  
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"a" gram paraffin of the melting point  $T_1$  is mixed with "b" gram of the melting point  $T_2$ , the melting point  $T_x$  of the resulting mixture is:

$$T_x = \frac{a \cdot T_1 + b \cdot T_2}{a + b} \quad \text{Example: } 100 \text{ parts paraffin } 62^\circ \text{ mixed with } 10 \text{ parts beeswax } 54^\circ =$$

$$T_x = \frac{100 \cdot 62 + 10 \cdot 54}{100 + 10} = 61. \quad \text{Heating raises the melting point about } 1^\circ, \text{ thus:}$$

$$T_x = \frac{100 \cdot 63 + 10 \cdot 55}{100 + 10} = 62.$$

Used paraffin is less contaminated by volatile substances. On the other hand, it contains tiny water droplets left over from the cooling procedure, as well as glycerin (boiling point  $290^\circ \text{ C.}$ ), which is used to prevent the block from sticking to the imbedding dish (see *B*).

#### PROCEDURE

*A. Fresh Paraffin.* (1) Fresh paraffin is weighed, put in a shallow enameled dish (as used in photography), and heated on an electric plate. At about  $80^\circ \text{ C.}$  benzol evaporates. If the liquefied paraffin begins to boil at  $100^\circ$  to  $110^\circ \text{ C.}$ , this proves the presence of water. In this case, splashing can be avoided by leaving the temperature below  $120^\circ \text{ C.}$  until all the water is evaporated. The paraffin is then heated to  $220^\circ \text{ C.}$  and kept at this temperature for about three to four hours. During this procedure disagreeable-smelling fumes escape; the paraffin, while absorbing oxygen, takes on a yellowish color. It is then cooled and left overnight. In the cooled paraffin no heterogeneous spots should appear. Otherwise the procedure should be repeated.

(2) Yellow beeswax is added in the proportion of 10 per cent of the weight of the paraffin. The mixture is kept at a temperature of  $180^\circ \text{ C.}$  for thirty to forty-five minutes, then filtered and cooled.

*B. Used Paraffin (Remainders of A).* (1) The block rests are rinsed in water and dried. (2) The paraffin is heated to  $180^\circ \text{ C.}$  for thirty to forty-five minutes, then filtered and cooled.

#### SUMMARY

A mixture of paraffin and beeswax is described which can be sectioned with ease, the melting point of which hardly differs from that of the original paraffin.

#### REFERENCE

Krause, R.: *Enzyklopaedie der Mikroskopischen Technik*, Berlin, 1926, Urban & Schwarzenberg.

## A SIMPLIFICATION OF BENHOLD'S TEST FOR AMYLOIDOSIS\*

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THE standard Congo red test, with or without acetone extraction, has two major disadvantages. (1) It is rather complicated, requiring one intravenous injection and two venipunctures and consuming more than one hour to perform. (2) There is a large range, say from 40 to 80 or 90 per cent dye retention, which is not diagnostic for amyloid disease but does not exclude it. This may possibly be due to the inability of slight amyloid deposits to fix a sufficiently large part of the Congo red injected.

It was observed during the performance of the standard test that in patients with amyloidosis the apparent dye content of the serum four minutes following the injection of Congo red was obviously considerably less than in normal patients.

In Fig. 1 the percentage of dye retention is plotted against time during the first hour following the intravenous injection of 10 c.c. of a 1 per cent solution of Congo red. Subject A had amyloidosis; Subject B did not. The calculations are based on the colorimetrically determined dye content and on the estimated total plasma of the respective patients. The vastly faster absorption of the dye in the amyloidotic patient is shown particularly in the early phase following injection. It would appear that this early phase allows a more reliable distinction between the presence and absence of amyloidosis. Since the estimation of the percentage retention of Congo red, as done in the standard test, increases the uncertainty of interpretation rather than adds a quantitative evaluation, the aim was to find a proper amount-time relation so that all persons not suffering from amyloidosis would show Congo red in the serum and patients with amyloidosis would not.

In preliminary experiments it was found that Congo red in a 0.00025 per cent solution can easily be demonstrated by the appearance of a blue color when one drop of this dilution is mixed with one drop of concentrated hydrochloric acid. When 2 c.c. of 1 per cent solution of Congo red are injected in a normal person weighing 150 pounds, the plasma (about 2,700 c.c.) would contain about 0.00074 per cent of Congo red, or three times the demonstrable concentration. Even with 10 or 20 per cent resorption during the first fifteen minutes, Congo red should still be demonstrable fifteen minutes following the injection.

The present modification is, then, based on (1) the rapid initial absorption of Congo red in the presence of amyloid, (2) the slow initial absorption of Congo

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red in persons without amyloidosis, (3) the easy demonstrability of Congo red in a dilution as low as 0.00025 per cent.

#### TECHNIQUE

Two cubic centimeters of a 1 per cent Congo red solution are injected intravenously, and after exactly fifteen minutes a few drops of blood are withdrawn from a finger into a suitable tube with capillary mouth (Widal tube). After the blood has clotted, it is centrifuged at slow speed and one or two drops of clear serum are removed with a capillary pipette and placed into a small white porcelain dish. One drop of undiluted hydrochloric acid is added with another capillary pipette. If the serum contains Congo red, a blue, quickly fading color, appears at the moment of mixing.

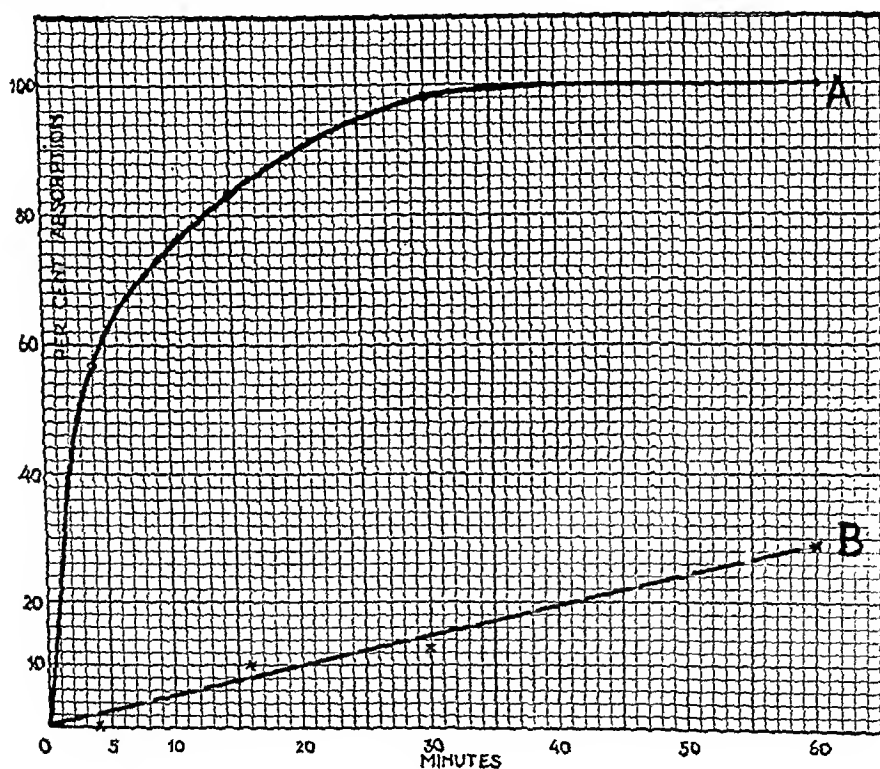


Fig. 1.

#### RESULTS

A total of 245 persons who presented no evidence of amyloid disease and in whom it was not suspected were examined with this technique. All showed Congo red in their serum, with the exception of one patient. With the 10 c.c. method this patient had given the results shown in Table I. On March 13, 1939, he was positive for amyloid according to the 2 c.c. method. He had massive albuminuria, a fixed urinary specific gravity between 1.017 and 1.020. Liver and spleen were not enlarged. At autopsy on Feb. 18, 1940, no amyloid was seen. The kidneys showed a few areas of hyaline degeneration of glomeruli, and the

spleen contained minute hyaline, rounded bodies. Microchemically these hyaline areas could not be identified as amyloid. Since this patient had massive albuminuria, he might have excreted Congo red through his kidneys, thus giving a false positive test. The urine was not examined for Congo red at the time of the test.

TABLE I

| DATE     | PER CENT |
|----------|----------|
| 2/24/38  | 15       |
| 10/20/38 | 100      |
| 12/30/38 | 40       |
| 4/ 7/39  | 44       |
| 4/11/39  | 33       |
| 9/18/39  | 75       |

A total of 33 patients with evidence of amyloid disease were tested, and all but one gave a positive test. The evidence for amyloid disease in these patients is as follows:

8: confirmation by autopsy.

32: 95 to 100 per cent Congo red retention by the standard test.

Many of these patients had the usual clinical and chemical evidence of amyloidosis.

The one patient with amyloidosis who yielded a negative test showed moderate amyloidosis of kidneys and spleen at autopsy on March 4, 1939. She had none of the usual clinical signs of amyloidosis, and a 10 c.c. Congo red test done on July 5, 1938, showed 20 per cent retention. The falsely negative 2 c.c. test was performed on Jan. 11, 1939.

#### DISCUSSION

According to this experience, the reliability of this modification is not less than that of the standard 10 c.c. method. However, many further observations are necessary to define its limit of reliability correctly.

This modification is so much simpler than the original test (requiring one venipuncture instead of three, and fifteen minutes observation instead of one hour) that it seemed justified to report it.

#### SUMMARY

1. A modification of the Congo red test for amyloidosis is reported.
2. It is simple in technique and requires only one instead of three venipunctures.
3. Its reliability, which needs further testing, appears to be of the same degree as that of the standard technique.

# A COMPARISON OF CAPILLARY AND VENOUS RED BLOOD CELL COUNTS AND HEMOGLOBIN DETERMINATIONS IN PATIENTS WITH PERNICIOUS ANEMIA IN REMISSION UNDER TREATMENT\*

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IT IS generally believed<sup>1</sup> that in adult man the red blood cell count and hemoglobin content of blood obtained by puncture of the ear or finger and that obtained by venepuncture without stasis is the same, although Haden and Neff<sup>2</sup> found lower values in the blood obtained from central vessels than from skin capillaries in infants. In a recent article De Marsh and co-workers<sup>3</sup> state in a footnote that they consistently find the sinus blood of infants to have lower values for red blood corpuscles and hemoglobin than blood obtained from puncture of the heel. Duke and Stofer<sup>4</sup> demonstrated that in 8 patients with pernicious anemia, 5 of whom were in relapse, capillary red blood cell counts averaged 17.6 per cent higher than venous counts, whereas such differences were not encountered in 5 normal persons or in 4 patients with "secondary" anemia.

In the course of an analysis of the effect of treatment in maintaining blood levels in 80 patients with pernicious anemia, it became apparent that a decided difference existed between the results of blood counts and hemoglobin determinations made on venous blood and on capillary blood. A total of 2,139 blood examinations were analyzed. Approximately two-thirds of the examinations were made on capillary blood during the first years of this study; since January, 1938, all determinations have been made on venous blood. From 14 to 46 separate examinations were made on each of the 80 patients over a period of from five to nine years (average 7.1 years). All but 5 patients had both capillary and venous determinations, although, as indicated above, these determinations were not made at the same time. All patients were under constant treatment and in remission. The arithmetic means of the venous and capillary red blood cell counts and hemoglobin determinations were calculated for *each* patient. The distributions of these means have been plotted in Fig. 1.

The mean capillary red blood cell count of the 80 patients was 4.690 millions per cubic millimeter, and the mean venous red blood cell count of 75 of these 80 patients was 4.444 millions per cubic millimeter, a difference of 0.246 millions per cubic millimeter, a value which is distinctly significant, there being less than one chance in 1,000 of its being accidental ( $t = 4.712$ ;  $n = 153$ ;  $p = < 0.001$ ).

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The mean of the hemoglobin determinations on capillary blood of the 80 patients was 91.8 per cent (15.6 Gm. per 100 c.c. = 100 per cent), and the mean of the hemoglobin determinations on venous blood of 75 of these 80 patients was 87.3 per cent. The difference 4.5 per cent is significant, there being less than one chance in 1,000 that it is accidental ( $t = 4.657$ ;  $n = 135$ ;  $p = <0.001$ ).

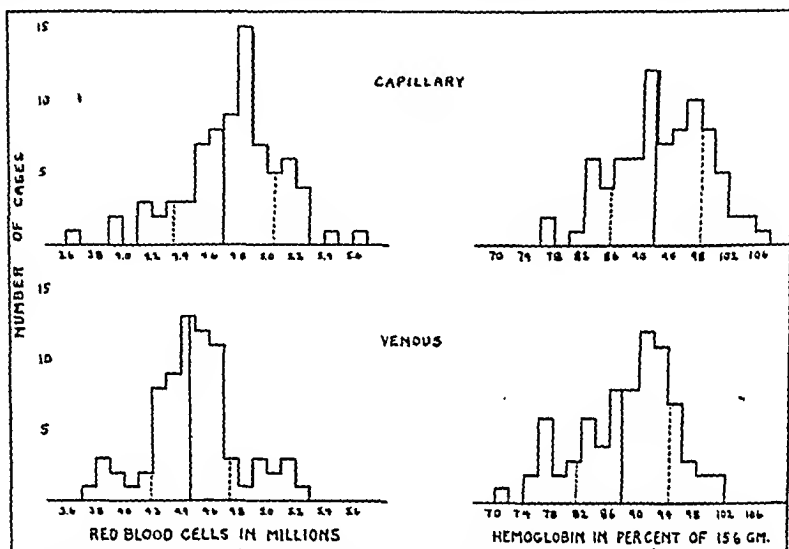


Fig. 1.—The distribution of the average capillary and venous red blood cell counts and hemoglobin determinations in 80 patients with pernicious anemia in remission while under treatment. The solid lines in each curve represent the mean value, and the dotted lines represent the standard deviations.

### CONCLUSION

Venous blood in patients with pernicious anemia under treatment has approximately 5 per cent less erythrocytes and hemoglobin than blood obtained from the capillaries. This may be true for normal persons as well, but we have insufficient data on this point.

It is a pleasure to acknowledge with thanks the technical assistance of Miss Eleanor Fleming, Miss Marjorie Jewell, Miss Florence Kelly, Miss Elizabeth King, Miss Harriet MacDonald, and Miss Charlotte Nicklin who performed the technical determinations.

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## A DEVICE FOR MARKING FIELDS ON MICROSCOPE SLIDES\*

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WHILE making a routine examination of histologic preparations, it is convenient, and sometimes necessary, to mark certain areas so that they may be readily found later. Among methods used for this purpose is one using a mechanical stage with vernier markings. A reading of the vernier markings will constitute a record of the location of the area to which future reference may be made. It is, of course, necessary that the same mechanical stage is used and that the slide stop remains unchanged. A second method utilizes a diamond point, placed in an eccentric position, attached to the rotating end of a blank objective. For operation, the regular objective of the microscope is replaced by the blank objective and, with the slide held firmly, the diamond point is lowered to contact the cover slip. A circle is described by turning the rotating end and scratching the cover slip with the diamond. Disadvantages of the method are that sometimes the cover slip is moved or broken and the permanent scratch mark may obliterate an area desired for study. A third and rather crude method consists simply of marking the area with a pen and India ink. A difficulty of this procedure is to center properly the desired area within the inked circle; usually several attempts are necessary to obtain the desired result. Failure of an attempt can be corrected by removing the ink mark with a dampened cloth.

The third method of pen and ink marking has been improved by incorporating a principle of the second method in a simple device that may be fitted in the place of an objective and act as a rubber stamp. It consists of a brass cylinder, knurled and threaded at one end to fit into the revolving nosepiece of a microscope. The other end is reduced to a cylindrical shaft,  $\frac{3}{16}$  inch in diameter and about  $\frac{3}{8}$  inch in length. A short length of rubber tubing is slipped over the cylindrical shaft and allowed to project a short distance beyond the end of the shaft. The gum rubber tube supplied with a blood cell counting pipette was used for this purpose. The free end of the rubber tube must be cut squarely across. The length of the entire device must be such that the free end of the tube will make contact with the surface of the slide when the microscope tube is racked down.

The marker may be fitted to an empty socket or a revolving nosepiece on the microscope and be ready for use at all times. For operation the area of a slide to be marked is centered in the field of the microscope, and the slide is allowed to remain in place on the stage of the microscope. The rubber tip

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is inked with the quill of an India ink bottle. The marking device is then swung into the position formerly occupied by the objective, and the body tube is gently racked down until the inked rubber touches the slide. A ring of ink will be stamped on the slide to encircle the area desired. A little care is necessary to avoid application of excess ink on the rubber tip.



Fig. 1.—Device ready for attachment to nosepiece of microscope. The over-all length is slightly more than that of the longest microscope objective.

The diameter of the ink ring can be varied by having a larger or smaller shaft. The marker now in use was made with a detachable tip. Other tips of different diameters may be used.

The device has been in use for some time and found to be extremely useful in marking areas of slides to be studied or photographed at a later time. The inked ring mark is quite permanent under ordinary conditions and yet can be readily removed with a damp cloth.

## CHEMICAL

### THE EXTRACTION OF PROTEINS FROM AQUEOUS SOLUTION BY MEANS OF EMULSIFICATION WITH CHLOROFORM\*

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DURING the past fifty years there have been occasional references in the literature to the ability of chloroform to precipitate proteins more or less quantitatively from aqueous solution.<sup>1-3</sup> It is remarkable that such a method has not received closer scrutiny, particularly in view of the claims made in some of the earlier papers as to the completeness of removal attained. The method, furthermore, deserves investigation in view of its obvious advantage in not leaving behind in the aqueous layer an excess of a reagent that is difficult to remove and sometimes objectionable. This paper reports some systematic studies designed to evaluate the advantages and limitations of the procedure.

On shaking protein solution with chloroform there is quickly formed a heavy emulsion, which settles out readily on mild centrifuging in a compact cake below the supernatant water layer. A very satisfactory separation of this layer results even without centrifuging. The supernatant liquid can readily be decanted without disturbing the heavy emulsion, and the protein can be recovered by evaporation of the chloroform at room temperature in vacuum. Any foaming can be readily checked by the addition of a small amount of amyl alcohol;<sup>1</sup> its use, and that of ethyl alcohol, as noted below, have the further advantage of greatly increasing the completeness of protein removal.

Various forms of this method have been used by Tsuchihashi<sup>4</sup> for the preparation of blood catalase extracts; by Zeile and Hellström<sup>5</sup> for the preparation of liver catalase extracts; by Meldrum and Roughton<sup>6</sup> for the preparation of protein-free carbonic anhydrase extracts from blood; by Sevag<sup>7</sup> for the isolation of carbohydrates from egg albumin and pneumococci; by Sevag, Lackman, and Smolens<sup>8</sup> for the isolation of the components of streptococcal nucleoproteins; and by Gurin, Bachman, and Wilson<sup>9</sup> for the purification of the gonadotropic hormone of pregnancy urines.

#### EXPERIMENTAL

Kjeldahl determinations of total nitrogen made on the original protein solution and on the supernatant liquid after separation from the emulsion were used as the criterion of protein removal. The fact that different criteria, such as qualitative protein tests, were used by some of the earlier investigators,

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Baker's isoamyl alcohol with boiling range of 128° to 132° C. was used. For the sake of brevity it will be referred to in the remainder of this paper simply as "amyl alcohol."

undoubtedly accounts for some differences in our conclusions. The percentage of free amino nitrogen to total nitrogen was used as the criterion of molecular size. In preliminary experiments, amino nitrogen was determined by the Sorensen method, but the work was later repeated using Van Slyke determinations. The relationship between ability to be precipitated by this method and molecular complexity will be discussed in detail below.

Colorimetric pH determinations were made in the preliminary experiments; the glass electrode was used in all final work on the effect of variation of pH.

A preliminary series of extractions of 0.5 per cent egg albumin solution, made with varying proportions of chloroform, showed that maximum removal is effected by a volume of chloroform 25 to 30 per cent that of the protein solution. Smaller proportions of chloroform reduced the percentage removal; higher proportions brought about no improvement. As a result the standard proportions adopted were 100 ml. of protein solution and 35 ml. of chloroform. To this were added 10 ml. of amyl alcohol when it became evident that this mixture produced the optimum results of any investigated. Later work showed that 95 per cent ethyl alcohol could be substituted equally well for the amyl alcohol.

TABLE I  
EFFECT OF TIME OF SHAKING ON PERCENTAGE OF EGG ALBUMIN REMOVED

| TIME<br>(MINUTES) | PERCENTAGE PROTEIN REMOVED                 |  |   |
|-------------------|--|--|---|
|                   | NATURAL pH<br>CHLOROFORM ALONE<br>USED (%) | ISOELECTRIC pH<br>CHLOROFORM ALONE<br>USED (%) | ISOELECTRIC pH<br>CHLOROFORM + AMYL<br>ALCOHOL USED (%) |
| 1                 | 4.5  |  | 73.5  |
| 3                 | 8.9  | 16.8   | 80.0  |
| 5                 | 18.4                                       | 22.0   | 78.5  |
| 7                 | 27.7                                       | 25.2   | 79.8  |
| 10                | 31.8                                       | 40.6   | 78.2  |
| 16                | 33.2                                       |  |   |
| 20                | 38.1                                       |  |   |

Comparisons of the effect of mechanical shaking versus hand shaking showed that one is as effective as the other. Previous investigators have used widely varying times of shaking, in some cases as much as ten or more hours. However, we find no evidence quoted in support of such lengthy periods. We find that the effect of increased time of shaking (by hand) depends upon two other factors: the proximity of the pH of the solution to the isoelectric point of the protein, and the presence of the added alcohol. Table I shows the relative percentages of the protein removed from approximately 1 per cent egg albumin solution under these different conditions. With chloroform alone and the protein not adjusted to its isoelectric pH, an irregular increase up to twenty minutes is observed. The use of isoelectric conditions increases somewhat the percentage removal but does not bring it to constancy within ten minutes. However, the use of both isoelectric pH and amyl alcohol gives practically constant removals from three minutes upward. For this reason, a five-minute period of vigorous shaking by hand was adopted.

Tests of the effect of shaking with successive portions of the emulsifying agent also showed marked differences between chloroform alone and chloroform plus 10 per cent amyl alcohol.

TABLE II  
EFFECT OF SUCCESSIVE EXTRACTIONS OF THE SAME SOLUTION (APPROXIMATELY 1 PER CENT EGG ALBUMIN) AT ISOELECTRIC PH

| NUMBER OF EXTRACTIONS | TOTAL PROTEIN REMOVED BY |                               |
|-----------------------|--------------------------|-------------------------------|
|                       | CHLOROFORM (%)           | CHLOROFORM + AMYL ALCOHOL (%) |
| 1                     | 15.4                     | 84.5                          |
| 2                     | 24.7                     | 86.8                          |
| 3                     | 32.4                     | 87.3                          |
| 4                     | 37.5                     |                               |
| 5                     | 41.4                     |                               |

In Table II are recorded the results of a series of successive extractions of the same 1 per cent egg albumin solution in which the two procedures are compared. Percentages of protein removed are cumulative. The much more rapid effect of the chloroform-amyl alcohol mixture is plainly evident.

Other organic liquids of limited miscibility with water have been suggested for this purpose, such as ethyl acetate by Marie,<sup>10</sup> and bromoform by Formanek.<sup>2</sup> For this reason, a series of such liquids was investigated, employing the foregoing procedure described for egg albumin solutions of slightly under 1 per cent concentration. In some cases the test was made both at the natural pH of the protein solution (about pH 6) and at the isoelectric point (pH 4.8). A series of these results is recorded in Table III. In all cases the amount of the emulsifying agent used was one-third the volume of the albumin solution with, in some cases, addition of 10 ml. of the auxiliary agent (ethyl or amyl alcohols) per 100 ml. of the protein solution.

These results show the practical superiority of the chloroform-amyl alcohol and chloroform-ethyl alcohol mixtures over all other solvents investigated. We have not been able to correlate the low and irregular recoveries obtained with other liquids with any other physical property, such as boiling point, surface tension, water solubility, dielectric constant, etc. The surprisingly high removal effected by ethyl acetate prompted an examination of some homologous esters of different alcohols and acids. As will be noted, no benefit resulted.

Little has been done to investigate the colloidal properties of the protein-chloroform emulsion. Sevag, Laekman, and Smolens<sup>8</sup> suggest a loose molecular combination or "adsorption compound." A few tests on our part showed that water is attracted to a fragment of the emulsion on a microscope slide and enters it whereas chloroform does not. This would seem to indicate that the external phase is aqueous.

Previous investigators have discussed the properties of the precipitated protein,<sup>3, 6-8</sup> the consensus of opinion indicating more or less denaturation. Our own results confirm this. Egg albumin, precipitated by the above-described procedure, gave a product which, after washing out the chloroform and amyl alcohol with ether and drying at room temperature, was quite water insoluble.

After several days' shaking with water, the amount dissolved was insufficient to give biuret or sulfosalicylic acid tests. It was also insoluble in 0.1 N acid or alkali, salt solutions, and alcohol. Dried in vacuum to constant weight it had a nitrogen content of 14.5 per cent. Other methods of drying the emulsified protein gave a product similarly insoluble. The only case in which the precipitated and dried product was water soluble was when proteose-peptone solutions had been used as the starting point. In this case the fraction precipitated was correspondingly low (see below).

Contamination of the precipitated protein by dissolved salts is very slight. Experiments in which sodium chloride (about 1 per cent) was added to the aqueous protein solution showed that the presence of the salt did not appreciably affect the efficiency of removal by chloroform-amyl alcohol and that about 99 per cent of the salt remained in the water layer after emulsification.

TABLE III

EFFICIENCY OF VARIOUS ORGANIC LIQUIDS AS PROTEIN PRECIPITANTS FROM 1 PER CENT EGG ALBUMIN SOLUTION AT BOTH NATURAL AND ISOELECTRIC pH (4.8)

| REAGENT                    | PERCENTAGE PROTEIN REMOVED |                |
|----------------------------|----------------------------|----------------|
|                            | NATURAL pH                 | ISOELECTRIC pH |
| Chloroform                 | 48.7                       | 52.3           |
| Chloroform-isoamyl alcohol | 80.4                       | 82.5           |
| Chloroform-ethyl alcohol   |                            | 83.5           |
| Chloroform-ethyl acetate   |                            | 71.7           |
| Bromoform                  |                            | 19.0           |
| Bromoform-isoamyl alcohol  |                            | 20.0           |
| Bromoform-ethyl alcohol    |                            | 24.6           |
| Isoamyl alcohol            |                            | 60.0           |
| n-Amyl alcohol             |                            | 64.9           |
| n-Butyl alcohol            |                            | 38.8           |
| Capryl alcohol             | 4.7                        | 12.2           |
| Ethyl ether                | 22.5                       | 33.3           |
| Dichlorethyl ether         |                            | 18.7           |
| Carbon tetrachloride       | 9.3                        | 10.0           |
| Benzene                    | 14.8                       | 15.9           |
| Chlorobenzene              |                            | 23.9           |
| Bromobenzene               |                            | 19.0           |
| Toluene                    | 15.5                       | 19.5           |
| Benzaldehyde               |                            | 48.6           |
| Isobutyl chloride          |                            | 19.5           |
| Ethyl acetate              | 65.8                       | 69.7           |
| Ethyl propionate           | 28.6                       | 29.9           |
| Ethyl butyrate             | 31.3                       | 33.7           |
| n-Butyl acetate            | 22.9                       | 35.6           |
| Isoamyl acetate            | 20.0                       | 27.4           |

*Effect of pH.*—The data in Table III indicate that in some cases considerable differences in percentage removal of egg albumin from solution result from adjustment of the solution to pH 4.8 (isoelectric point). The column of values for the "natural pH" is included to illustrate the irregularities resulting when the protein solution is not adjusted to as near its isoelectric point as possible. In most cases, albumin solutions made from dried commercial preparations showed pH values of from 6.5 to 7.5. In some of the earlier papers,<sup>2, 8</sup> causal reference is made to different recoveries having been obtained at different acidities, although Zeile and Hellström<sup>5</sup> claim that the separation of hemoglobin from catalase is unaffected by pH variations between 4.5 and

9.0. We have not investigated hemoglobin in this regard but have made a systematic study of the effect of pH variations on percentage removal of egg albumin and serum albumin. Preliminary experiments were carried out using colorimetric determinations after titration with varying amounts of acid or alkali. Uniform extractions with either chloroform or chloroform-amyl alcohol were run on these solutions, and the percentage removal of the protein was determined as described previously. One outstanding difference between the use of chloroform alone and of the chloroform-amyl alcohol mixture was observed. In all cases, maximum precipitation occurs at or near the isoelectric point. However, with chloroform alone this maximum is a comparatively low one, whereas with either alcohol added, the curve, starting from about the same level, rises rapidly and sharply to a much higher maximum. At pH values, for example, two units above or below the isoelectric pH, only small differences were observed between the use of chloroform alone or with amyl alcohol; at the isoelectric pH, however, the difference was 30 per cent or more.

TABLE IV

VARIATION BETWEEN pH OF AQUEOUS PROTEIN SOLUTION AND PERCENTAGE PRECIPITATION BY THE CHLOROFORM-AMYL ALCOHOL PROCEDURE

Approximately 1 per cent solutions of all proteins were used.

| BLOOD ALBUMIN<br>(WILL) |                         | BLOOD ALBUMIN<br>(KAHLBAUM) |                         | EGG ALBUMIN<br>(BAKER) |                         | EGG ALBUMIN<br>(BAKER)* |                         |
|-------------------------|-------------------------|-----------------------------|-------------------------|------------------------|-------------------------|-------------------------|-------------------------|
| pH                      | %<br>PRECIPITA-<br>TION | pH                          | %<br>PRECIPITA-<br>TION | pH                     | %<br>PRECIPITA-<br>TION | pH                      | %<br>PRECIPITA-<br>TION |
| 4.49                    | 27.0                    | 3.63                        | 9.6                     | 4.25                   | 41.8                    | 4.20                    | 47.3                    |
| 5.24                    | 84.4                    | 3.85                        | 10.2                    | 4.49                   | 58.4                    | 4.45                    | 54.9                    |
| 5.95                    | 95.3                    | 4.11                        | 13.7                    | 4.60                   | 68.6                    | 4.75                    | 82.1                    |
| 6.48                    | 78.0                    | 4.42                        | 18.5                    | 4.67                   | 71.1                    | 4.79                    | 82.5                    |
| 7.01                    | 39.4                    | 4.82                        | 48.4                    | 4.70                   | 74.2                    | 4.88                    | 87.0                    |
|                         |                         | 5.00                        | 71.3                    | 4.76                   | 77.4                    | 5.00                    | 88.4                    |
|                         |                         | 5.25                        | 72.3                    | 4.82                   | 78.2                    | 5.30                    | 89.4                    |
|                         |                         | 5.85                        | 27.5                    | 4.85                   | 78.6                    | 6.06                    | 62.0                    |
|                         |                         | 6.62                        | 7.6                     | 4.90                   | 81.5                    | 9.78                    | 31.1                    |
|                         |                         | 7.25                        | 6.9                     | 5.00                   | 84.9                    |                         |                         |
|                         |                         | 8.45                        | 6.9                     | 5.23                   | 84.2                    |                         |                         |
|                         |                         | 8.95                        | 4.8                     | 5.95                   | 55.8                    |                         |                         |
|                         |                         |                             |                         | 7.35                   | 34.1                    |                         |                         |
|                         |                         |                             |                         | 9.45                   | 19.7                    |                         |                         |

\*Chloroform-ethyl alcohol used.

A more accurate determination was then made of the curves of pH versus percentage protein removal for both egg albumin and blood albumin, all pH determinations being made by means of the glass electrode. The results are shown in Table IV. The sharp maximum obtained is obvious for both blood albumin and egg albumin, but it will be observed that this maximum is uniformly higher by several tenths pH than the isoelectric point usually accepted for egg albumin. We have not as yet redetermined the isoelectric point of this material and can, therefore, offer no explanation. However, it may be stated that this maximum roughly approaches the isoelectric point of the protein.

*Effect of Molecular Size of Protein.*—Preliminary experiments indicated that this method of precipitation is somewhat selective as regards the molecular

size of the protein. Using the ratio of free amino nitrogen to total nitrogen as a measure, it was evident that the larger aggregates were precipitated, leaving in the aqueous layer a residue of such high free amino nitrogen to total nitrogen ratio that it often failed to respond to qualitative precipitation tests for proteins. This circumstance no doubt accounts for the fact that some of the earlier investigators claimed complete precipitation.

TABLE V

RELATIONSHIP BETWEEN PERCENTAGE PRECIPITATION OF PROTEINS AND THE CHANGE BETWEEN FREE AMINO NITROGEN TO TOTAL NITROGEN RATIO OF THE ORIGINAL PROTEIN SOLUTION AND THAT REMAINING UNEXTRACTED IN THE AQUEOUS LAYER  
All solutions at isoelectric pH.

| SOURCE OF MATERIAL          | CONC. OF PRO-<br>TEIN PER 100<br>ML. (GM.) | PERCENTAGE<br>PRECIPITA-<br>TION | RATIO: FREE AMINO N TO<br>TOTAL N $\times$ 100 |                             |
|-----------------------------|--|----------------------------------|--|-----------------------------|
|                             |  |                                  | IN ORIGINAL<br>SOLUTION                        | IN<br>SUPERNATANT<br>LIQUID |
| Egg albumin, crystallized*  | 0.92                                       | 98.8                             | 3.63   | 19.64                       |
| Egg white, fresh            | 0.73                                       | 83.8                             | 2.51   | 5.72                        |
| Egg albumin, Baker's scales | 0.85                                       | 81.1                             | 4.80   | 7.61                        |
| Egg albumin, Baker's powder | 0.78                                       | 82.5                             | 5.40   | 11.72                       |
| Egg albumin, Merck's scales | 1.16                                       | 80.3                             | 4.12   | 9.45                        |
| Serum albumin, Kahlbaum     | 0.75                                       | 49.7                             | 7.24   | 10.30                       |
| Serum albumin, Will         | 0.74                                       | 92.3                             | 6.76   | 8.89                        |
| Serum albumin, old sample   | 0.77                                       | 17.8                             | 5.27   | 5.73                        |
| Dog serum, diluted          | 1.08                                       | 15.2                             | 7.43   | 8.62                        |
| Proteose-peptone, Witte     | 4.54                                       | 6.8                              | 15.33  | 18.83                       |
| Proteose-peptone, Difco     | 4.52                                       | 2.7                              | 17.46  | 19.23                       |

\*Kindly prepared by Dr. Samuel Gurin, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

To investigate this point, comparisons were made of the free amino nitrogen to total nitrogen ratio in the original protein solution and in the supernatant aqueous layer after precipitation. This comparison was applied to blood albumin and egg albumin from different sources as well as to some commercial proteose-peptone mixtures, from which very little precipitation could be accomplished. In Table V are given the results obtained from a number of different proteins. In these determinations the chloroform-amyl alcohol mixture was used throughout. Free amino nitrogen to total nitrogen ratios cannot be recorded for the precipitated protein because of its insolubility (see above). The only exception encountered was in proteose-peptone solutions. For example, the commercial proteose-peptone solutions are listed in Table V, which showed a very low percentage precipitation and a proportionately small rise in the free amino nitrogen to total nitrogen ratio, yielded a small amount of precipitate which was sufficiently water soluble to make possible Kjeldahl and Van Slyke determinations. These samples showed a corresponding decrease in the ratio from that of the original solution.

The data in Table V are presented as typical of a large number of such determinations. It is evident from these data that the higher the original ratio of free amino nitrogen to total nitrogen, the smaller the percentage of the total material removed by this treatment and the less selective the removal. Some samples of serum albumin purposely chosen were old and already partly hydrolyzed. The behavior of such samples, in comparison with some egg



albumin samples of a lower ratio of free amino nitrogen to total nitrogen is plainly evident. A number of such determinations were also made on protein solutions at pH values other than the isoelectric. Aside from small quantitative irregularities the same conclusion holds: in general, the higher the proportion of the protein precipitated, the more marked the change in free amino nitrogen to total nitrogen ratio between the original solution and the residual material.

It is evident that chloroform precipitation, while in many cases a useful tool for protein removal, has distinct limitations which should be recognized. Its chief advantage lies in the fact that the aqueous solution, from which the protein is being removed, remains uncontaminated by an excess of the precipitating reagent, as is the case when precipitation is accomplished by tungstic acid, trichloroacetic acid, etc. Furthermore, it is sometimes an advantage not to have changed the pH of the aqueous solution, and the precipitated protein, although denatured, is free from metallic contamination.

The chief disadvantage to be recognized is the failure of the method to effect a quantitative removal of the protein even under optimum conditions. It should, therefore, not be used if a small percentage of residual nitrogen in the aqueous solution is objectionable. Since, however, the unprecipitated fraction is of simpler structure with a high ratio of free amino nitrogen to total nitrogen, this may not always be an objectionable feature. The use of the chloroform procedure by other investigators,<sup>4,9</sup> as mentioned above, exemplifies this.

Although most of our data have been collected on protein solutions approximating 1 per cent concentration, a number of determinations have been made on egg albumin solutions, ranging from 0.5 per cent to about 8 per cent. We have not found that the completeness of removal is appreciably affected by variations in concentration within these limits.

#### CONCLUSIONS

Studies of the removal of proteins from solution by chloroform emulsification have led to the conclusion that the procedure is a useful one for certain purposes and when certain limitations are recognized. The following is recommended as the optimum procedure:

The protein, in water solution, should be as near its isoelectric pH as possible. The most advantageous proportions studied were 100 volumes of the protein solution, 35 volumes chloroform, and 10 volumes of 95 per cent ethyl or of isoamyl alcohols. The mixture should be shaken vigorously for five minutes in a container of about twice its volume, then centrifuged for about five minutes at a speed of 2,000 to 2,500 r.p.m. Mechanical shaking is unnecessary.

Successive treatments of the aqueous solution with new portions of chloroform and alcohol cause no significant increase in the amount of protein removed.

Similar tests of a number of organic liquids of limited solubility in water have brought to light none approaching the chloroform-alcohol mixture in efficiency. The nearest approach is ethyl acetate. Homologues of this ester have all given much lower percentage removals.

The precipitated protein is denatured by the process to the point of being insoluble in water, salt solutions, and dilute acid and alkali. The only exception to this is in the very small amount of precipitate obtained from proteose-peptone solutions.

The presence of small amounts of salts in the protein solution does not appreciably affect the emulsification process nor the purity of the denatured protein.

When chloroform alone is used as the emulsifying agent, the curve of percentage removal *versus* pH reaches a low maximum at or near the isoelectric point of the protein. With the alcohol added this maximum becomes sharp and much higher.

In any protein solution investigated, the fraction of higher molecular weight is preferentially precipitated, leaving in solution a fraction with a ratio of free amino nitrogen to total nitrogen much higher than in the original solution. The lower this ratio is originally, the more effectively does this procedure remove the protein.

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# THE QUANTITATIVE DETERMINATION OF INDOXYL COMPOUNDS IN URINE\*

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## INTRODUCTION

FOR nearly a quarter of a century there has been a general absence of interest in the significance of the urinary excretion of indole derivatives. The doctrine established by tradition that urinary indoles are derived from putrefactive processes in the intestines apparently has made further investigation of this subject unprofitable.

Recent work, however,<sup>1, 2</sup> has revived earlier observations<sup>3-5</sup> that the presence of indoles may be demonstrated with unusual ease in the urine of pellagrins. This observation merits further investigation, since the doctrine of the intestinal derivation of urinary indoles was formulated at a time when biochemical and medical science was unaware of the importance of nutritional deficiency.

The most familiar indole derivative that may be encountered in urine is indican (indoxyl potassium sulfate). Several methods for the estimation of this substance in urine have been proposed in the past. Most of these methods are crude and inadequate to meet the demands of modern quantitative investigation. Part of the indole excreted in urine may occur in a form which is responsible for the uroscopin reaction,<sup>7</sup> but for which no method of quantitative estimation has as yet been developed. This study is concerned with a discussion of the methods available for the quantitative determination of indican in urine, and the presentation of a procedure, modified from Sharlit's method,<sup>6</sup> which has been found satisfactory for quantitative purposes.

## EXPERIMENTAL

The qualitative detection of indican in urine depends on the reaction obtained by adding concentrated hydrochloric acid and an oxidizing agent (ferrie chloride or calcium hypochlorite) to urine. By this means indican undergoes oxidation and polymerization to form the characteristic blue pigment indigo, which may then be extracted from aqueous solution with chloroform. There have been various attempts in the past to utilize this reaction in the quantitative colorimetric estimation of indican. However, there are certain objections to methods based on this procedure.

(1) After the addition to the urine of hydrochloric acid and an oxidizing agent, a single extraction with chloroform may be insufficient to remove all the

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indigo formed, particularly when the indigo is formed slowly, as sometimes happens. Occasionally, it is possible to extract more indigo after the reaction mixture has been subjected to an initial extraction and then allowed to stand for some minutes.

(2) The addition of a fixed amount of the oxidizing agent to some urine specimens results in overoxidation of the indican and a diminished yield of indigo, while in other urine specimens incomplete oxidation occurs.

(3) The oxidation by ferric chloride or calcium hypochlorite does not always yield indigo blue. Indigo red (indirubin), which may have a different color intensity, is sometimes obtained.

(4) The recovery of a known amount of indican from indican-free urine is frequently far from quantitative.

(5) Difficulties arise in the preparation of a suitable standard for comparison. The intensity of color obtained in the chloroform solution of indigo extracted from urine is frequently more intense than that of a saturated solution of synthetic commercial indigo. In our experience recrystallized synthetic indigo has a maximum solubility of approximately 2 mg. per 100 ml. of chloroform at room temperature. When a saturated solution of synthetic indigo is evaporated to dryness and then taken up in strong sulfuric acid, a marked intensification of color results, indicating that the color reaction of indigo can be modified by such procedure. Great caution is, therefore, necessary in the use of any artificially prepared standard for purposes of comparison with unknown indigo solutions.

These difficulties are largely overcome by Sharlit's technique.<sup>6</sup> This method makes use of the formation of a condensation product of indoxyl with thymol in the presence of persulfates. The condensation product is extracted from aqueous solution by forming within the reaction mixture itself the organic solvent ethyl trichloroacetate, which slowly settles out, carrying the pigment down with it. Under these conditions both the reaction and the subsequent extraction can be carefully controlled. This procedure, when modified as described below, has been adopted as the method of choice.

#### PROCEDURE

Ten milliliters of unfiltered urine preserved under toluene are placed in a volumetric flask and diluted to 100 ml. with distilled water. Five milliliters of this diluted urine are measured into a 15 ml. graduated centrifuge tube. The reagents are added in the following order: 0.5 ml. of 1 per cent potassium persulfate solution, 0.5 ml. of 1 per cent thymol in 95 per cent ethyl alcohol, 5 ml. of 25 per cent trichloroacetic acid in concentrated hydrochloric acid. At the same time another tube is prepared for a blank determination; urine and reagents are added in the same quantities *except that thymol-free alcohol* is employed. After the solutions have been mixed, each tube is placed in a boiling water bath until the ethyl trichloroacetate has separated out, and the aqueous layer is essentially free from pigment. This ordinarily requires about twenty minutes. The tubes are then centrifuged briefly and as much as possible of the supernatant aqueous layer is removed with a pipette. The trichloroacetate layer,

together with the small volume of aqueous layer remaining in the centrifuge tubes, is taken up in sufficient glacial acetic acid to bring the volume to the 5 ml. mark; the solutions are then transferred to special tubes for use in a Klett-Summerson photoelectric colorimeter, and are thoroughly mixed by pouring back and forth between the centrifuge tube and the colorimeter tube. The intensity of color in the acetic acid solutions is read immediately in the photoelectric colorimeter, using a No. 540 filter. The logarithmic scale reading given by the blank determination is subtracted from the reading of the unknown solution. The concentration of indican in micrograms per milliliter of urine is calculated as given below. If the reading on the colorimeter is less than 200, or more than 400 scale units, greater accuracy is achieved by repeating the test, using appropriately greater or smaller amounts of urine.

#### DISCUSSION OF METHOD

*Preservation of the Urine.*—Estimations have been carried out on twelve- and twenty-four-hour specimens of urine. Although it is usually stated that the indican reaction must be performed on freshly passed urine, we have found that the addition of a few milliliters of toluene to the urine when first passed is sufficient to maintain the values obtained by this method for at least twenty-four hours. As pointed out by Sharlit, formaldehyde should not be used as a preservative.

*Amounts of Reagents.*—Using 0.5 ml. of urine, it was found that the amount of potassium persulfate could be varied considerably without affecting the results of the determination. We have used 0.5 ml. of a 1 per cent solution. The results were found also to be unaffected by increasing the volume of alcoholic thymol solution employed. In practice 0.5 ml. of a 1 per cent alcoholic solution of this reagent is sufficient. The 25 per cent trichloroacetic acid in concentrated hydrochloric acid used by Sharlit<sup>8</sup> in his estimation of indican in plasma was employed successfully in urine estimations.

*Time Necessary for the Various Stages.*—It was not found necessary to allow the tubes to stand for fifteen minutes to cool after removal from the water bath, as recommended by Sharlit. Nor was any advantage gained by allowing the tubes to stand at various stages of the test, as Townsend<sup>9</sup> recommended. Considerable saving of time is achieved by the omission of these steps.

*Colorimetry.*—Townsend<sup>9</sup> has shown that the spectrum of the indoxyl-thymol condensate exhibits maximum light absorption at about 540 m $\mu$ . A No. 540 filter was, therefore, used in the colorimeter. The use of a Klett-Summerson photoelectric colorimeter has definite advantages over visual colorimetry. The personal factor is eliminated in making the readings. Furthermore, since solutions of the indoxyl-thymol condensate obey Beer's law reasonably well within the limits of the concentrations recommended in this procedure, the readings obtained on the logarithmic scale of the colorimeter can be converted directly into terms of a concentration of indican by simple multiplication of the reading by the same factor over the recommended length of scale.

*The Standard.*—The conversion factor was experimentally determined by carrying out the estimation on an indican solution of known concentration.

Obviously the validity of the standard depends upon the purity of the specimen of indican used for this purpose. It is possible that indican obtained from various commercial sources may vary appreciably in its degree of purity. This is suggested by the fact that the figures given by Townsend<sup>9</sup> for the level of indican in normal plasma unaccountably disagree with those given by Sharlit.<sup>8</sup> This discrepancy may be partly explained by differences in their standards, due to different degrees of purity in the specimens of indican they employed. For this reason, particular precautions were taken to ensure the purity of the indican used in obtaining the standard. A specimen of indican,<sup>\*</sup> manufactured by Fraenkel and Landau, was purified by recrystallization. The temperature of decomposition was checked and found to be 180° C. Micro-determinations for sulfur and nitrogen yielded the results given in Table I. These values show satisfactory agreement with the theoretical values when the errors of the methods are considered.

TABLE I

| FOUND    |               | THEORETICAL   |
|----------|---------------|---------------|
| Nitrogen | 4.6 per cent  | 5.2 per cent  |
| Sulfur   | 12.7 per cent | 11.9 per cent |

Further tests showed that this specimen of indican gave standard readings which agreed well with those obtained by Sharlit who used a specimen from a different source. He employed a solution of cobalt sulfate as a standard for use in a comparison colorimeter, and stated that a 1.5 per cent solution of  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  matched the color given by 2 ml. of acetic acid solution containing indoxyl-thymol condensate derived from 0.0086 mg. of indican. We found that under our conditions 50  $\mu\text{g}$  of indican gave the same colorimeter reading as a 3.5 per cent solution of  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ . This agrees with results of Sharlit to within 3 per cent.

*Calculation of Results.*—The colorimeter readings obtained with different amounts of indican were determined experimentally under the conditions of the test using standard aqueous solutions of indican. The results are given in Table II.

TABLE II  
DETERMINATION OF THE CONVERSION FACTOR FOR INDICAN

| INDICAN IN $\mu\text{g}$<br>PER 0.5 ML. | NUMBER OF<br>OBSERVATIONS | AVERAGE COLORIMETER<br>READING | CONVERSION<br>FACTOR |
|---|---------------------------|--------------------------------|----------------------|
| 25                                      | 4                         | 137                            | 0.182                |
| 50                                      | 13                        | 262                            | 0.190                |
| 75                                      | 5                         | 392                            | 0.191                |
| 100                                     | 9                         | 492                            | 0.203                |
| 125                                     | 6                         | 600                            | 0.208                |

It will be seen from the figures in Table II that the factor necessary to convert colorimeter readings into terms of the amount of indican present varies somewhat over the range employed; or, in other words, when colorimeter read-

\*Obtained through the courtesy of Dr. S. R. Townsend.

ings are plotted against concentrations of indican, the resulting calibration curve is not absolutely linear, as required to fulfill Beer's law exactly. However, the differences between the factors for each concentration of indican are so small that for practical purposes one factor may be used throughout. We have employed the factor 0.38 to convert colorimeter readings into terms of micrograms of indican per milliliter of solution. This factor is obtained from the equation:

$$\text{Factor} = \frac{\text{Indican in micrograms}}{\text{Colorimeter reading} \times \text{volume of indican solution}} = \frac{50}{262 \times 0.5} = 0.38,$$

substituting the values for the point of greatest experience, Table II. Use of this single factor for all concentrations introduces a possible error of not more than 5 per cent when the colorimeter readings lie between 200 and 400 scale units. The twenty-four-hour excretion of indican is calculated as follows:

$$24\text{-hour excretion of indican in mg.} = \text{Colorimeter reading} \times \frac{0.38}{1,000} \times \text{volume of urine.}$$

Since it is possible that there may be slight differences between individual colorimeters of this type, we suggest as a check the use of a 3.5 per cent  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  solution to determine the colorimeter reading which should be given by 50  $\mu\text{g}$  of indican under the conditions of this test.

*Recovery of Indican Added to Urine.*—We have found that on adding a known amount of indican to urine the recovery is quantitative.

*Blank Determination.*—Sharlit did not feel it necessary to carry out a blank determination as recommended here. However, we have found that in certain urine specimens (notably in specimens obtained from cases of liver disease) high blank readings are sometimes obtained with the use of thymol-free alcohol. This high blank is due to nonspecific substances which on oxidation by persulfate form colored products soluble in ethyl trichloroacetate. Such substances might include urochrome and perhaps bile products. Contrary to Sharlit's statement, we have found that preliminary treatment of the urine with basic lead acetate may sometimes result in a significant reduction in the colorimeter reading, due to the removal of such interfering pigments. In theory a small part of the blank estimation may be due to indican itself, since in the presence of persulfate it could be converted to indigo which, dissolved in the trichloroacetate, may increase the reading on the colorimeter. However, in our experience the addition of indican to a number of urine specimens has resulted in no significant increase in the blank reading. In practice, therefore, the blank is subtracted from the reading of the unknown without correcting for a small error which occasionally may be introduced by the additional color added to the blank by the conversion of indican to indigo by persulfate.

If the persulfate is also omitted from the blank determination, very much higher readings may be obtained, which indicates that persulfate plays some part in the decolorization of pigments which would otherwise interfere still further with the estimation.

It is evident that any substance will interfere in this test if it can form a colored condensate with thymol in the presence of persulfate, which in turn

is soluble in ethyl trichloroacetate and has an absorption band in the region of  $540 \mu\mu$ . Our own experience and that of Sharlit indicate that it is highly improbable that urinary constituents other than indican will contribute significantly to such color production. Sharlit showed that of a number of substances tested, only glucose, formaldehyde, and tryptophane produced any color under these conditions, and then only in insignificant amounts. In addition, we have investigated the effect of added indole and indole-3 acetic acid on the test. Two-tenths milligram of each of these substances when added to samples of urine taken for test failed to produce any significant increase in the colorimeter reading. It seems, therefore, that this reaction is sufficiently specific to exclude unsubstituted indole, and its simple substitution products, such as indole acetic acid. Hence it is probable that the test is, in fact, specific for indoxyl and its salts and just possibly a few other derivatives of indole so closely related chemically that they are indistinguishable from indican.

#### SUMMARY

The need for a reinvestigation of the significance of the urinary excretion of indole derivatives has prompted a consideration of the various methods available for the estimation of indoxyl compounds in urine. It has been found that Sharlit's method, with the modifications contributed by this investigation, yields quantitative results.

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## A MODIFICATION OF THE COLORIMETRIC PHOSPHORUS DETERMINATION FOR USE WITH THE PHOTOELECTRIC COLORIMETER\*

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THE two most widely used colorimetric methods for the determination of phosphorus are those of Fiske and Subbarow,<sup>1</sup> and of Kuttner and Cohen,<sup>2</sup> with minor adaptations of the techniques for specific purposes. The Benedict-*Theis*<sup>3</sup> modification of Briggs'<sup>4</sup> method, although highly praised for its accuracy by Bodansky,<sup>5</sup> cannot be used in the presence of phosphoric esters because boiling, which is a part of the procedure, will hydrolyze them.

Both the Kuttner-Cohen and the Fiske-Subbarow methods have certain advantages as well as inconveniences. The one great advantage of Kuttner and Cohen's method is the excellent stability of the color. However, this advantage is more than counterpoised by two serious drawbacks: first, the extreme sensitivity of the method to interfering substances and to minimal impurities in the reagents; second, the narrow range of acid concentration within which colorless blanks and proportionate readings are obtained. A slight deviation from this concentration results in colored blanks or erratic readings, or both. A third much less serious disadvantage is that the stannous chloride reagent has to be diluted fresh daily. The Fiske-Subbarow method is far less sensitive to interfering substances and to the concentration of acid. However, it still requires the use of three different molybdate solutions for the use with samples containing various amounts of acid. The second drawback of the method is that the aminonaphtholsulfonic acid reducer does not keep well. Even in a well-stoppered bottle it becomes increasingly yellowish brown, and at the same time its reducing power slowly declines, especially if the sulfite used in preparing the solution is not absolutely fresh. A 14-day-old solution may give 10 to 15 per cent lower readings than a freshly prepared one, as found in our experiments. In addition, sometimes for no apparent reason it will precipitate in the test tube, making the liquid so turbid that it cannot be read without filtration. The third drawback of the Fiske-Subbarow method is that the intensity of the color produced increases continuously for several hours. If a visual colorimeter is used this will cause no complication, since the standard will darken at the same rate. However, with a photoelectric colorimeter, an instrument which is superseding the older visual types at a fast rate, the results may be considerably off if readings are not taken at a strictly specified time.

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An attempt was made to develop a method which combines the advantages of the methods mentioned, with elimination of their drawbacks.

#### EXPERIMENTAL

In a first series of experiments the Benedict-Theis, Fiske-Subbarow, and Kuttner-Cohen methods were compared with respect to (1) their acid tolerance, and (2) the stability of the color produced. All readings were obtained with a photoelectric colorimeter.

Acid tolerance is defined as the permissible range of final acid concentration within which the method gives uniform readings at the optimum time suggested by the deviser of the method. It was found that within wide limits of molybdate concentration (0.1 to 1 per cent  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ) the acid tolerance of each method depends only on the proportion between molybdate and sulfuric acid in the final mixture. Acid tolerance will be expressed by the permissible range of cubic centimeters of normal sulfuric acid per 10 mg. of sodium molybdate under the conditions mentioned.

| Acid Tolerance        |                  |
|-----------------------|------------------|
| Benedict-Theis Method | 0.9 to 2.2 c.c.  |
| Fiske-Subbarow Method | 1.1 to 2.1 c.c.  |
| Kuttner-Cohen Method  | 1.3 to 1.45 c.c. |

If the amount of acid present in the mixture is below the lower limit of tolerance, the readings will be too high and the blanks may be colored; if the amount of acid is above the upper limit, the readings will be too low.

As shown by the data, the range of acid tolerance of the Kuttner-Cohen method is very narrow. With the Fiske-Subbarow method the final concentration of acid is 2 c.c. per 10 mg. of molybdate. This is so close to the upper limit of tolerance that they realized the necessity of using special mixtures (molybdate II and molybdate III) with samples that contain additional free acid. Hydroquinone, used in the Benedict-Theis method, has an excellent acid tolerance. We found that boiling, an undesirable feature of this method, is unnecessary if the concentration of hydroquinone is increased to 1 per cent and that of the bisulfite is cut to 3 per cent. Although the color is somewhat slow to develop, it is extremely stable for a long period of time. This slow development of color is the main objection of Fiske and Subbarow to the Briggs method. However, it is hard to see why this should be considered a shortcoming as long as the method is satisfactory otherwise. In fact, slow development of color with sustained stability is a decided advantage, especially when a large number of determinations have to be done with a photoelectric colorimeter.

However, one of the objections of Fiske and Subbarow to hydroquinone is a valid one. It is the production of a brownish shade with molybdates.

The stability of the colors obtained with the methods mentioned is given in Table I.

Some other substances, part of which were tried previously by Fiske and Subbarow, were examined in order to find one that would give stable solutions and colorless blanks. Three of them, 2:4-diaminophenol hydrochloride (com-

mercial names, amidol, acrol), methyl-p-aminophenol sulfate (commercial names, metol, elon, graphol, etc.), and p-phenylenediamine were found to meet all requirements, in spite of the fact that Fiske and Subbarow were not satisfied with the first two. We are unable to explain why they did not find them satisfactory since they did not state their reasons.

Of the three substances mentioned, *elon* is the one of choice on account of its low price and its excellent keeping qualities. Unlike aminonaphtholsulfonic acid, it is readily soluble in a bisulfite solution. It can be kept almost indefinitely either in the crystalline form or in solution with 3 per cent sodium bisulfite.\* The blanks produced with it are colorless. It is very insensitive to many common interfering substances. The following substances in the amounts stated per 15 c.c. of final mixture did not produce appreciable deviations in the readings in our set-up:

|                      |         |
|----------------------|---------|
| Sodium fluoride      | 15 mg.  |
| Sodium oxalate       | 15 mg.  |
| Sodium citrate       | 15 mg.  |
| Sodium nitrite       | 3 mg.   |
| Sodium nitrate       | 450 mg. |
| Sodium chloride      | 500 mg. |
| Ammonium sulfate     | 500 mg. |
| Ferric chloride      | 15 mg.  |
| Trichloroacetic acid | 750 mg. |

In the case of large amounts of silicates special precautions, which will be mentioned later, have to be taken.

The acid tolerance of *elon* is 0.9 to 2.2 c.c. This considerable range of acid tolerance permits the use of a single molybdate-sulfuric mixture for all kinds of samples, with almost no regard for the amount of free acid they may contain. For instance, if in the set-up to be described later 5 c.c. of the molybdate-sulfuric reagent is used, an amount of acid equivalent to 0.5 c.c. of concentrated sulfuric acid will be tolerated.

The intensity of the color is practically the same as that obtained in the Fiske-Subbarow method. The stability of the color is given in Table I. The data were obtained by first calibrating the curves at the optimum times of reading, as specified by the authors or as found by us. Values at other times were read from these curves. The sample contained 0.04 mg. of phosphorus. Readings at optimum times are in italics.

TABLE I  
1/100 MG. OF PHOSPHORUS

|                                 | 5 MIN.   | 10 MIN.  | 30 MIN.     | 60 MIN.     | 120 MIN.    |
|---------------------------------|----------|----------|-------------|-------------|-------------|
| Kuttner-Cohen method            | <i>4</i> | <i>4</i> | <i>4</i>    | <i>4</i>    | <i>4.06</i> |
| Fiske-Subbarow method           | 3.92     | <i>4</i> | <i>4.15</i> | <i>4.25</i> | <i>4.5</i>  |
| Hydroquinone (room temperature) | 3.2      | 3.65     | <i>4</i>    | <i>4</i>    | <i>4</i>    |
| <i>Elon</i>                     | 3.35     | 3.82     | <i>4</i>    | <i>4</i>    | <i>4</i>    |

On the basis of the afore-mentioned data and of the constancy and proportionality of the readings obtained with *elon*, we are of the opinion that its use offers definite advantages over previous methods.

\*Müller<sup>6</sup> uses *elon* in a 5 per cent solution of neutral sodium sulfite. This solution keeps very poorly. The reading time proposed by Müller (fifteen minutes) is too low.

The following method was found to be simple and dependable. Its results in many hundred determinations on biological materials (serum, tissues, phosphatase) were compared with those obtained with the Fiske-Subbarow and Kuttner-Cohen methods, and proved to be in excellent agreement:

*Solutions required.*

1. 10 N sulfuric acid. Pour 282 c.c. of concentrated sulfuric acid into 600 c.c. of water. Cool and make up to 1,000 c.c.

2. Molybdate-sulfuric reagent. Mix 2 parts of a 5 per cent solution of sodium molybdate ( $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ), 1 part of 10 N sulfuric acid, and 1 part of water.

3. Reducing solution. Dissolve 1 Gm. of *clon* in 100 c.c. of a 3 per cent solution of sodium bisulfite.

*Procedure.* The method has been worked out for use with photoelectric colorimeters only.

1. Calibration of the curve. Prepare a standard solution of monobasic potassium phosphate, containing 0.01 mg. of phosphorus per cubic centimeter (110 mg. of the salt in 2,500 c.c., with 1 c.c. of concentrated sulfuric acid). Measure into each of 7 test tubes or volumetric flasks, as the type of the colorimeter may require, 2.5 c.c. of the molybdate-sulfuric reagent. Add 2 c.c. of the phosphorus standard to the first tube, and 1 c.c. more to each consecutive tube. The last tube will contain 8 c.c. of the standard. Add 1 c.c. of *clon* solution to each of the test tubes and fill them up to 15 or 25 c.c., respectively.\* Read within from forty-five to ninety minutes. Use a red filter with transmission limits of about 600 and 750  $\text{m}\mu$ . Plot readings on a semi-logarithmic graph paper. The readings should fall in a straight line.

If a range of much higher phosphorus values is required, the method of calibration will remain the same, except for proportionately larger amounts of reagents and final volumes.

2. Determination of phosphorus in sample. Measure 2.5 c.c. (or, if the sample is very acid, as after wet ashing, 5 c.c.) of molybdate-sulfuric reagent into a test tube or flask. Add about 5 c.c. of water. Add a measured aliquot of the sample, calculated to have a phosphorus content within the reading limits. Measure 1 c.c. of *clon* solution into the tube and fill up to the mark. Read as before.

If the amount of phosphorus in the sample is entirely unknown, one may proceed in the following way: Place 2.5 c.c. of the molybdate-sulfuric reagent in the test tube and dilute it with about 5 c.c. of water. Add first a small aliquot, say 1 c.c., of the sample, followed by 1 c.c. of *clon* solution. Wait a few minutes. With some experience it will be easy to judge whether the intensity of the color is within the range of readings. If it is, dilute to the usual volume. If the solution looks too pale, another measured aliquot may be added until the color seems to be of about the right intensity. On the other hand, if the solution is too dark, it should be diluted to a greater final volume

\*With the Evelyn colorimeter the final volume should be 25 c.c.; with colorimeters using more narrow test tubes, such as the Lumetron apparatus, 15 c.c.

(up to 8 times). In our experiments we found that if such diluted solutions are read from a curve calibrated at a smaller volume, a correction of 1.75 per cent has to be added to the values for each 100 per cent of diluent; for instance, with a curve calibrated at 25 c.e., a reading at 50 c.e. requires a correction of +1.75 per cent; at 100 c.e., a correction of  $3 \times 1.75$  or +5.25 per cent.

In the case of the presence of large amounts of silicate add 0.5 c.e. of 10 N sulfuric acid for each 2.5 c.e. of the molybdate-sulfuric reagent to the solution before adding the sample in order to depress the reduction of silicomolybdic acid. Take readings between forty-five and fifty minutes. The results of this slight modification of the *elon* method compare very favorably with those obtained with the Fiske-Subbarow technique. It was found that the latter method is far more sensitive to silicates than the data given by the authors would indicate.

Table II shows the results of the Fiske-Subbarow method, of the original, and of the modified *elon* method in the presence of sodium silicate. All samples contained 0.05 mg. of phosphorus in 15 c.e. of the final mixture. To one series of the tubes 2 mg. of sodium silicate ( $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ ) were added; to the other series 10 mg. Readings were made in 1/100 mg. of phosphorus.

TABLE II

|                               | 5 MIN. | 10 MIN. | 30 MIN. | 45 MIN. | 60 MIN. | 90 MIN. |
|-------------------------------|--------|---------|---------|---------|---------|---------|
| Fiske-Subbarow                |        |         |         |         |         |         |
| Control                       | 4.2    | 5       | 5.22    | 5.28    | 5.35    | 5.45    |
| 2 mg. silicate                | 4.2    | 5.1     | 5.7     | 5.86    | 6.2     | 6.5     |
| 10 mg. silicate               | 4.2    | 5.8     | 7.5     | 8.2     | 9.1     | 11.1    |
| <i>Elon</i> , original        |        |         |         |         |         |         |
| Control                       | 4.3    | 4.6     | 4.9     | 5.0     | 5.0     | 5.0     |
| 2 mg. silicate                | 6.3    | 7.25    | 9.25    | 11.1    | 14.5    |         |
| 10 mg. silicate               | 14.5   |         |         |         |         |         |
| <i>Elon</i> , modified        |        |         |         |         |         |         |
| $\text{H}_2\text{SO}_4$ added |        |         |         |         |         |         |
| Control                       | 4.3    | 4.5     | 4.8     | 5.0     | 5.0     | 5.0     |
| 2 mg. silicate                | 4.5    | 4.7     | 5.0     | 5.0     | 5.1     | 5.16    |
| 10 mg. silicate               | 4.6    | 4.8     | 5.0     | 5.1     | 5.3     | 5.5     |

If the sample is alkaline, it must be neutralized or, even better, acidified with trichloroacetic or dilute sulfuric acid, since the acidity of the molybdate-sulfuric reagent is close to the lower limit of tolerance.

## SUMMARY

The advantages and drawbacks of the Fiske-Subbarow and the Kuttner-Cohen methods for the colorimetric determination of phosphorus are evaluated. The use of *elon* as a reducing agent is suggested. A new method, adapted for use with the photoelectric colorimeter, is described. This method combines the advantages and eliminates the handicaps of the methods mentioned.

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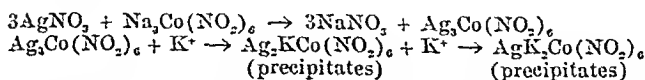
## ADAPTATION OF THE SILVER COBALTINITRITE METHOD FOR POTASSIUM TO THE PHOTOELECTRIC COLORIMETER\*

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THE silver cobaltinitrite potassium method was introduced by Breh and Gaebler (1930) as an improvement of the cobaltinitrite method of Kramer and Tisdall (1921). This procedure has been modified and its capabilities have been studied by Robinson and Putnam (1936), Truszkowski and Zwenner (1937), Ismail and Harwood (1937), Weichselbaum, Somogyi, and Rusk (1940), and Harris (1940).

The main advantages of this method over the more standard chloroplatinate method are: simpler analytical technique, lack of interference of sodium ion, decrease in the time required for an analysis, and the relatively small cost of the reagents used. However, the method has the serious disadvantage that it is based on reactions which do not occur in a strictly stoichiometrical basis.

Silver cobaltinitrite is formed by the interaction of silver nitrate and sodium cobaltinitrite. Silver cobaltinitrite forms highly insoluble salts with potassium. These reactions can be represented as follows (Burgess and Kamm, 1912):



As these reactions indicate, the composition of the precipitated potassium salt is not fixed and may vary under different conditions of precipitation; hence the potassium content of the precipitate cannot be calculated on a stoichiometric basis unless the precipitation is carried out under rigidly controlled conditions.

Chloride, silver, nitrite, and ammonium ions will interfere with potassium determination by this method. The chloride ion reacts with the silver of the silver cobaltinitrite reagent to form insoluble silver chloride. The amounts of silver and nitrite present influence the composition of the precipitated

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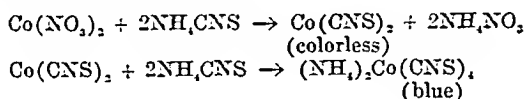
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potassium salt (Weichselbaum and co-workers, 1940). If these two ions are present in relatively large amounts, they will precipitate out as silver nitrite crystals. The ammonium ion reacts with silver cobaltinitrite reagent similarly to potassium, forming an insoluble complex, which unless the ammonia has been previously removed would be determined as potassium.

The silver potassium cobaltinitrite complex decomposes and goes into solution when exposed to hot acid or alkali. The amount of silver, nitrite, or cobalt in the resulting solution can be determined and serves as an indirect measure of the amount of potassium in the solution from which the complex was precipitated.

The cobalt ion in relatively nonaqueous solutions, such as 95 per cent alcohol or acetone, reacts with ammonium thiocyanate according to the following reactions (Heinz, 1929; Steffen, 1932):



Breh and Gaebler (1930) used this reaction as a means of determining the potassium colorimetrically. This paper deals with the adaptation of this colorimetric method to the photoelectric colorimeter.

#### METHODS

The light transmission of alcoholic ammonium cobalt thiocyanate solutions from potassium analyses of standard potassium sulfate solutions was examined spectroscopically. The color of these cobalt solutions was found to be a mixture of red and blue, since the solutions absorb a relatively narrow band of light between 5,900 and 6,360 Å.U. The type of photoelectric colorimeter\* used in this study measures the intensity of light transmitted through the solutions being analyzed, and in this sense might better be called a photometer. The instrument attains a high degree of sensitivity, and in a sense becomes a colorimeter when used with filters which absorb all light outside the absorption ranges of the solutions to be analyzed. A compound filter, which transmitted light between 5,800 and 6,560 Å.U., was found suitable for use with these cobalt solutions.

The silver cobaltinitrite reagent and the precipitation procedure used in the potassium analyses were as described by Truszkowski and Zwemer (1937).† By means of a stopcock-controlled siphon, equipped with a capillary tip bent up at the end and beveled at the same slope as the inside of the pointed tip

\*The Evelyn Photoelectric Colorimeter, manufactured by the Rubicon Company, Philadelphia, Pa., was used in this study. The Klett-Summerson Photoelectric Colorimeter, manufactured by the Klett Manufacturing Company, New York, has been used in more recent experiments.

†In more recent experiments the precipitation procedure and wash reagent of Harris (1910) have been used, and 1.5 c.c. of 7 per cent sulfuric acid have been substituted for nitric acid as the solvent for the precipitated potassium complex. Such solutions made up to 10 c.c. photoelectric colorimeter show a straight line relationship between concentration and the Klett galvanometer reading. The best sensitivity has been attained with a combination of Corning filters Nos. 245, 965, and 978.

centrifuge tube\* in which the precipitation of the silver potassium cobaltinitrite complex is carried out, it has been found possible to withdraw the supernatant fluid completely enough so that the required number of washings and consequent centrifugings can be reduced from three to one without detectable loss in accuracy.

The procedure developed for the preparation of the potassium complex for photometric analysis was as follows: The supernatant wash fluid above the silver potassium cobaltinitrite complex was removed as completely as possible with the capillary siphon described above. One cubic centimeter of a 7 per cent nitric acid solution was added, and the precipitate was dissolved by careful heating over a microburner. After cooling, the volume was made up to the 15 c.c. mark of the calibrated centrifuge tube with a freshly prepared 2 per cent ammonium thiocyanate solution in 95 per cent ethyl alcohol. A blank consisting of 1 c.c. of 7 per cent nitric acid made up to 15 c.c. with the thiocyanate solution was also prepared. The centrifuge tubes were closed with rubber stoppers previously washed with a nitric acid-alcohol solution with the same acid and alcohol content as the blank. The tubes were centrifuged for three minutes to remove any suspended particles. The solutions were then poured into the photometer tubes, the photometer tube shield placed at the 10 c.c. position, the zero point adjusted, and the rheostats set so that the galvanometer deflected to the 100 mark with the blank solution. The deflections of the cobalt solutions were then recorded. The photometer readings for alcoholic ammonium cobalti-thiocyanate solutions prepared in this manner and tightly stoppered will remain constant for several hours at room temperature and for days if kept at temperatures below 10° C.

The photometer was calibrated by running several series of analyses on known potassium sulfate solutions with potassium contents from 0.05 to 0.50 mg. by steps of 0.05 mg. A calibration curve was constructed by plotting the log of the galvanometer deflections against the potassium content.

## RESULTS

The accuracy obtained in potassium analyses of dog whole blood and plasma with this method, using an ordinary colorimeter, has been previously reported (Wood, Collins, and Moe, 1940). Calculations based on 251 duplicate analyses indicated that the probable error of a single analysis was 1.2 per cent, or 2.3  $\mu$ g of potassium per cubic centimeter of plasma. The photoelectric colorimeter, if it is to be satisfactory for use with this method, must have a sensitivity which will allow detection of potassium content differences of less than 3  $\mu$ g. The standard deviation of a single photoelectric potassium analysis was calculated from 74 triplicate analyses of standard potassium sulfate solutions. The

\*Very pointed tip centrifuge tubes similar to the Keys (1937) chloride tube, but made with a small glass foot to prevent perforation of shield cushions during centrifugation, have been found suitable for the accuracy of the procedure and greatly increase it.

†It was found that the alcoholic cobalt thiocyanate solution was not stable if the silver potassium cobaltinitrite complex was dissolved in 20 per cent nitric acid, as described by Brehl and Gaebler. In the course of an hour or so, depending on the temperature, this solution turns green, and a yellow precipitate forms, probably due to the transformation of the thiocyanate to perthiocyanogen as a result of the oxidizing action of the nitric acid (McAlpine and Soule, 1933). This difficulty can be avoided by the use of 7 per cent nitric acid as the dissolving agent.



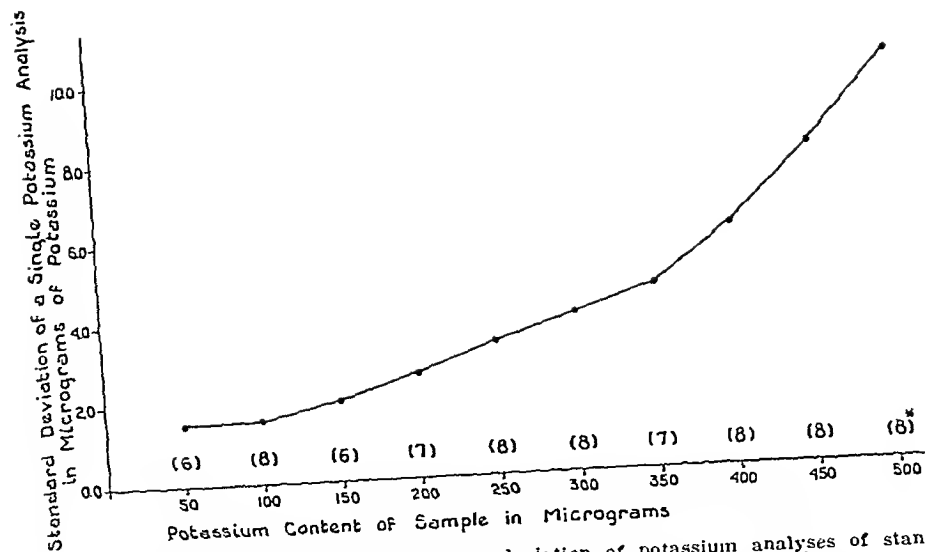


Fig. 1.—The relationship between the standard deviation of potassium analyses of standard potassium sulfate solutions and the potassium content of the sample.

\*Number of triplicate analyses upon which each point is based. The calculated standard deviation for each point was corrected for the size of the sample.

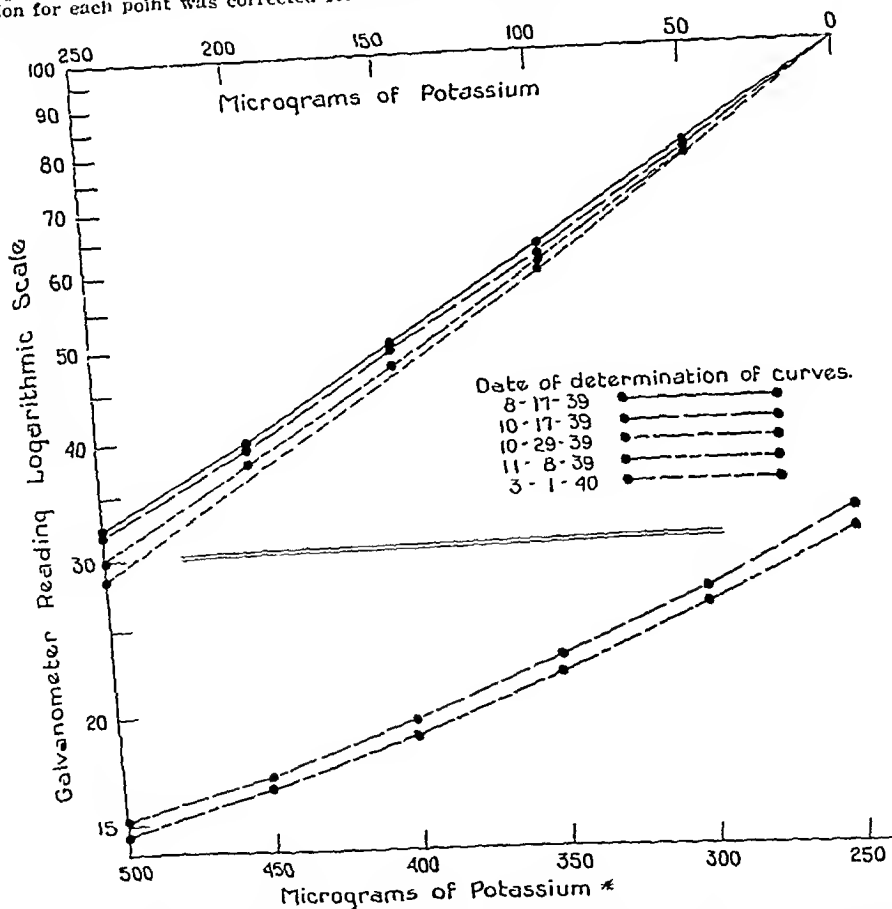


Fig. 2.—Potassium analyses of standard potassium sulfate solutions: Variation of photometer calibration curves with aging of the sodium cobaltinitrite reagent (color: ammonium cobalthiocyanate blue).

\*All standard potassium sulfate solutions were made up by dilution from the same stock solution. Each point is based on the averages of from 3 to 9 separate potassium analyses.

standard deviations have been plotted against the amount of potassium in the sample (Fig. 1). The average probable error in samples ranging from 50 to 500  $\mu\text{g}$  of potassium was  $\pm 1.1$  per cent (0.9-2.0). This probable error for potassium analyses is acceptable for most biologic studies.

The photoelectric colorimeter calibration curve (Fig. 2) in the region from 50 to 300  $\mu\text{g}$  of potassium was found to approach a straight line, the theoretical prediction for such an instrument. The slope of the calibration line in this region is such that 2.5 to 3.0 galvanometer divisions represent 10  $\mu\text{g}$  of potassium. Since the galvanometer can be read accurately to somewhat less than one division, the sensitivity of the instrument will allow detection of concentration differences of less than 3  $\mu\text{g}$  of potassium, or in terms of plasma concentrations, 0.3 mg. per 100 c.c.

Plasma potassium analyses by this method are carried out on 1 c.c. amounts of plasma; hence potassium analyses of from 50 to 300 micrograms are equivalent to plasma potassium concentrations of from 5 to 30 mg. per 100 c.c. This concentration range far exceeds the normal range of plasma potassium concentration found in man or in laboratory animals.

It was found that as the sodium cobaltinitrite reagent ages, the galvanometer deflections obtained from analyses of the same standard solution gradually decrease, i.e., an apparent increase in the potassium content of the standard solution. This finding was checked by determining a series of four calibration curves at intervals during a period of over six months. All standard potassium sulfate solutions used for the determination of these curves were made up by dilution from the same stock solution. The silver cobaltinitrite reagent was made up fresh on the day of its use by the addition of 5 c.c. of 40 per cent silver nitrate to 100 c.c. of the same stock solution of sodium cobaltinitrite reagent. The silver cobaltinitrite reagent was filtered by suction through a No. 4 sintered glass filter just prior to its use. A family of four separate calibration curves was obtained (Fig. 2). This change in the calibration curve apparently indicates that the cobalt content of the precipitated silver potassium cobaltinitrite complex increases as the sodium cobaltinitrite reagent ages. This constitutes a serious source of error in the use of the photoelectric colorimeter for this method. This source of error can be avoided to a large extent by running duplicate analyses of a standard potassium solution (containing 0.25 mg. of potassium) along with the analyses of unknown solutions. Since the calibration curves approach straight lines, the potassium content of the unknown solutions can be determined with reasonable accuracy by interpolation between the calibration curves on the basis of the deflection obtained with the standard solution.

The average results obtained for the potassium content of blood and tissue by the use of this method agree well with those reported from other laboratories using other methods (Wood and Moe, 1942; Wood, 1942).

Use of the photoelectric colorimeter is much less laborious and time-consuming than the use of the ordinary colorimeter. By means of the method described it is easily possible for a single analyst to complete fifty potassium determinations within ten hours, omitting the time required for precipitation of the potassium complex.

## SUMMARY

(1) The adaptation of the silver cobaltinitrite method for potassium to the photoelectric colorimeter is described.

(2) The method is devoid of intricate technical procedures and substantially decreases the time required for a potassium analysis.

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# MEDICAL ILLUSTRATION

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## AGAR COMPOSITIONS FOR MOLDING: THE TECHNIQUE OF COMPOUNDING AND USING\*

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THE compounding and technique of using agar compositions for obtaining impressions of human tissue have been discussed at considerable length in previously published articles.<sup>1,2</sup> Recently a number of articles have appeared which add materially to the information on this subject. Most notable among these is the review by Paffenbarger,<sup>3</sup> who considered in detail commercial agar preparations. This author has perfected devices for testing compositions as to their strength under pressure, their elasticity, and their flowing qualities. He presents a set of general specifications for making agar compositions suitable for use in dentistry as a material for intraoral impressions. However, he does not explain how agar compositions may be compounded or changed to suit specific cases. It is the purpose of our paper to include a discussion of this important subject.

Since the appearance of the afore-mentioned articles, Kanfman<sup>4</sup> has devised a face mask of brass tubing through which cold water may be forced to facilitate rapid cooling of the negative, so that it may be removed from the tissue surface in a shorter period of time than was required previously. In this paper we shall also discuss the technique of preparing these molds in such a manner as to reduce the setting time of the agar composition.

In discussing the composition of agar molding materials, a basic formula may be considered. It should be realized that this formula is flexible and can be changed to suit specific requirements. Methods for changing the formula will be given following the reason for using each chemical.

|                      |                     |
|----------------------|---------------------|
| Agar                 | 4 ounces            |
| Water                | 100 ounces          |
| Zinc oxide           | 1 ounce             |
| Oxyquinoline sulfate | 10 grains           |
| Cellucotton          | $\frac{1}{2}$ ounce |
| Cotton               | 15 grains           |

Agar is the basic substance. When mixed with water it liquefies completely at 100° C. and does not jellyfy or start to set until it cools to about 40° C. Paffenbarger has found this setting point to vary from 36° to 44° C. in different batches of agar. It is this property of agar that makes it suitable as a molding material for use on living human tissue. The more serviceable

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\*From the Department of Art, University of Maryland School of Medicine, Baltimore.

composition should set at or near 44° C. If it sets below body temperature, 37° C., it is less satisfactory for use on living human skin. Agar differs greatly from gelatin or glue in this respect. It will adhere closely to the melting and setting points when mixed with water in spite of high or low concentrations. The amount of water in a gelatin or glue mixture plays a more important part on the temperature of its setting or jellying point. The higher the water content, the lower will be its setting point, and vice versa.

According to Paffenbarger, manufacturers report that they find it impossible to procure agar which is uniform from hatch to hatch. Agar from different species of seaweed from various parts of the world, or from different parts of the same country, shows varying characteristics.<sup>5</sup> Therefore, it becomes necessary to refine the crude product before it can be used as an impression material. We have found that the commercial powdered agar has less strength in solution than either the granular or shredded agar. However, the powdered agar goes into solution more readily. The higher the agar content, the stronger the composition will be, but its flowing qualities will be reduced accordingly. The agar composition must possess a viscosity that will permit easy flow into the smallest details to obtain accurate impressions, such as the fingerprint pattern. If the flow is too sluggish, air bubbles and lap or border lines will be formed in the mold, which in turn will produce a defective cast. The agar content, as well as each of the other ingredients, must be balanced against one another to produce a composition suitable for making intraoral molds, molds of the skin surface, and molds of inanimate objects. For example, a mold of the gum surface is made by pressing a tray filled with agar composition against the gums. Its fluidity is more viscous because the pressure exerted in a comparatively small space has a tendency to reduce the possible formation of air bubbles. A composition suitable for application with a brush to the human skin, such as a face or hand, should be less fluid, since it must flow by gravity without pressure into every detail. However, it must be sufficiently viscous to remain where it is placed in adequate quantities to obtain a mold of the desired thickness. A less viscous composition may be poured over an inanimate protoplasmic specimen, such as a part of the viscera, or a nonprotoplasmic specimen, such as a plaster cast placed in a walled area. In many cases the viscosity of the agar may be controlled by the water content of the mixture. In other words, the same mixture may be used for all three purposes if the water content is changed to suit the specific case. However, this is not the ideal method. The mixture should be compounded to suit definite requirements, because the agar content of a mixture often determines its setting strength or elasticity. Other ingredients, such as an excess of alum and iron sulfate, will reduce this strength despite the small amount of water used.

Water is an important factor in the plasticity and friability of agar compositions, both in the liquid and set states. The water content must be balanced against all other ingredients. Regardless of the other ingredients, if there is too much water in the mixture it will lack strength and elasticity. The agar content imparts these desirable properties to the mixture. The remaining ingredients offset difficulties that can and do arise when an agar-water mixture is used for molding.

*Zinc oxide*, talc, whiting, and similar nonsoluble earths and minerals mix physically with agar but have little chemical effect upon the composition as a whole. They give the mixture bulk and weight. Their main use is to change the electrolytic action of a setting plaster cast that is made in an agar mold, or to absorb any substance that may be excreted from the agar during and after setting. Such a secretion can cause a softening of the surface of a plaster cast made in an agar mold. In other words, if these fillers or absorbing agents are not included, a plaster cast made in an agar mold does not set properly. There are other substances, such as borax, sodium chloride, and sorbitol which have a chemical action on agar compositions, materially toughening them and at the same time reducing the plasticity or flowing qualities of agar compositions as a whole. Borax may be substituted for zinc oxide; it causes the resulting mixture to be more elastic if the cast is to be of wax. Such a mold has a detrimental effect on the plaster cast. However, it is possible to balance the borax or sorbitol against the zinc oxide, talc, or similar earth pigments to prevent this softening or "skinning" of the resulting plaster cast, and at the same time produce a more elastic mold. In fact, concentrated sorbitol-agar mixtures have a strength that is a thousand times greater than that produced by ordinary commercial agar molding compositions. The latter compositions serve their purpose well in the *cire-perdue* or lost wax casting process as an intermediate step in making metal casts of sculptures. These mixtures will have to be perfected for use as a molding material for plaster casts.

*Oxyquinoline sulfate* is probably the best preservative for agar compositions because it prevents mold growth over a long period of time. It is not as volatile as thymol, nor is it as toxic as phenol or sodium fluoride, all of which have been used without complete success. Sodium fluoride is quite toxic in large quantities, but it has its place as a preservative for colloidal jellies that are applied to inanimate nonprotoplasmic subjects. Oxyquinoline sulfate is used in such small proportion that it has no effect on the plasticity of the agar in the jelly state.

*Cellucotton* and *cotton* may be grouped together, since they have the same effect on agar mixtures, the effect being purely physical and not chemical. When these fibers are dispersed throughout an agar composition, they have a mechanical influence on the mixture, thereby holding it together. The cotton fibers are much longer than those of the cellucotton, which is a wood product. The amount of cotton fibers used must be kept low, for they tend to collect on the spoon during stirring and form in knots or clots throughout the mixture. These two substances, especially the cellucotton, increase the internal friction of the mass in both the liquid and set states. In other words, they give it body and reduce its flowing properties or plasticity.

Some manufacturers have incorporated rubber cement or para rubber dissolved into one of its solvents, such as benzine, into their agar compositions. After considerable experience in carrying out over two thousand experiments with such mixtures, we have come to the conclusion that the rubber, even when incorporated properly, is not only valueless in the mixture but is one of the most difficult ingredients to disperse evenly throughout the mass. It mixes only physically and not chemically, and provides thousands of tiny rubber

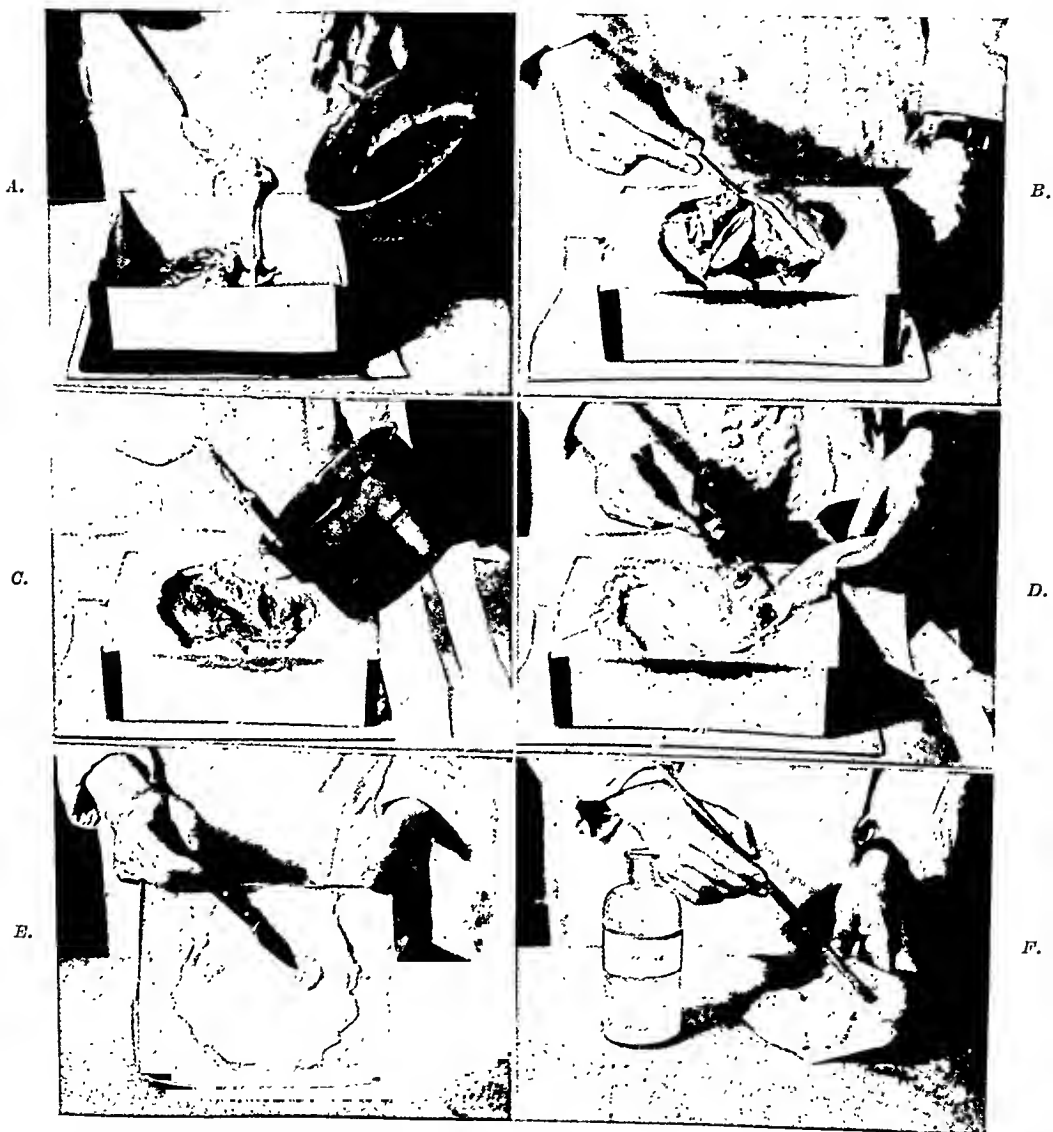


Fig. 1.—Making an agar mold of an inanimate object. An animate object, such as a hand, may be made in the same manner if a retaining wall of clay or cardboard is placed around the hand. A better method requiring less agar composition is shown in Fig. 3.

A. A cardboard or, preferably, a glass box is made as a retaining wall. The agar composition is poured into the box and allowed to set.

B. After setting, the specimen is removed.

C. The wax positive composition is poured or painted into the agar mold. If it is poured, the mold is turned until the composition covers all surfaces.

D. After the composition reaches a thickness of  $\frac{1}{8}$  inch, gauze is applied with melted wax to reinforce the mold.

E. Pieces of wood are put in place to serve as anchors for mounting the specimen on a wooden base. The remaining mold is poured with melted wax. If the specimen is to be hollow, latter method requires less wax.

F. After the wax reproduction is removed, it is given a coating of shellac-alcohol fixative to make it suitable for coloring. If this fixative is not applied, the wax-resin mixture of the positive will mix with the oil paints used in coloring.

bands that in themselves exert less mechanical influence in holding together the particles than does the same amount of cellulocotton. We are inclined to believe that the commercial manufacturer includes rubber purely for its psychologic effect on the buyer. It is reasoned that since rubber is tough and elastic, it

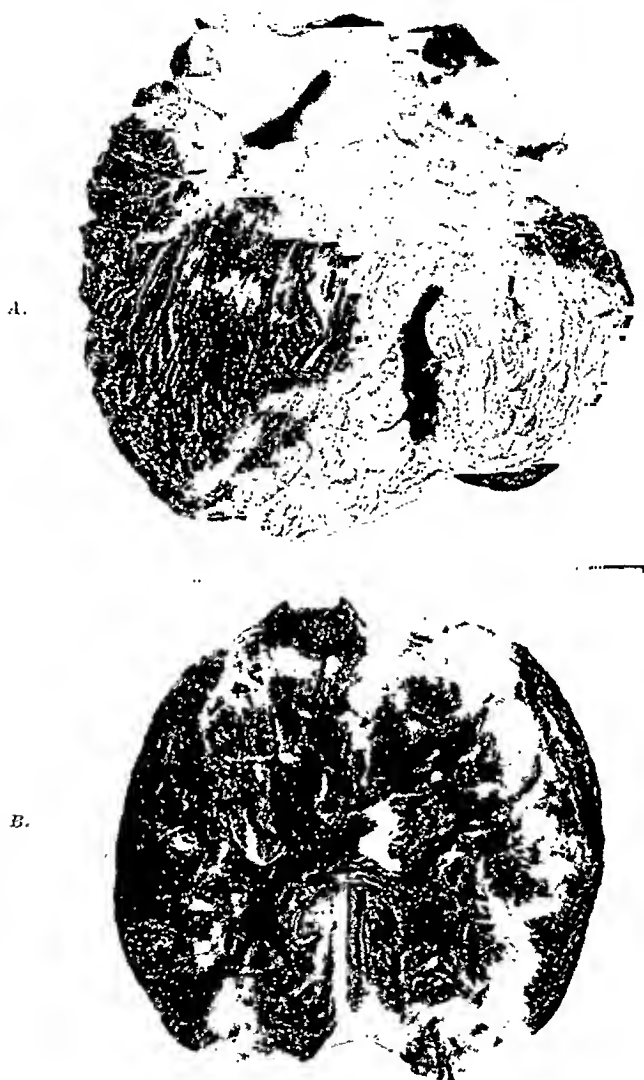


Fig. 2.—The finished wax-resin casts.

A, Wax specimen of an opened heart that was made in the agar mold seen in Fig. 1.

B, Wax cast of a kidney made in the same manner.

should impart these qualities to agar compositions, but we have found no evidence of such effects. Our tests and analyses have shown that the commercial products containing rubber were less elastic than those without it.

*Technique.*—The basic formula mentioned in this text can be used for the various types of molding specified. However, as in all types of art work, the manner in which the formula is compounded and used determines the



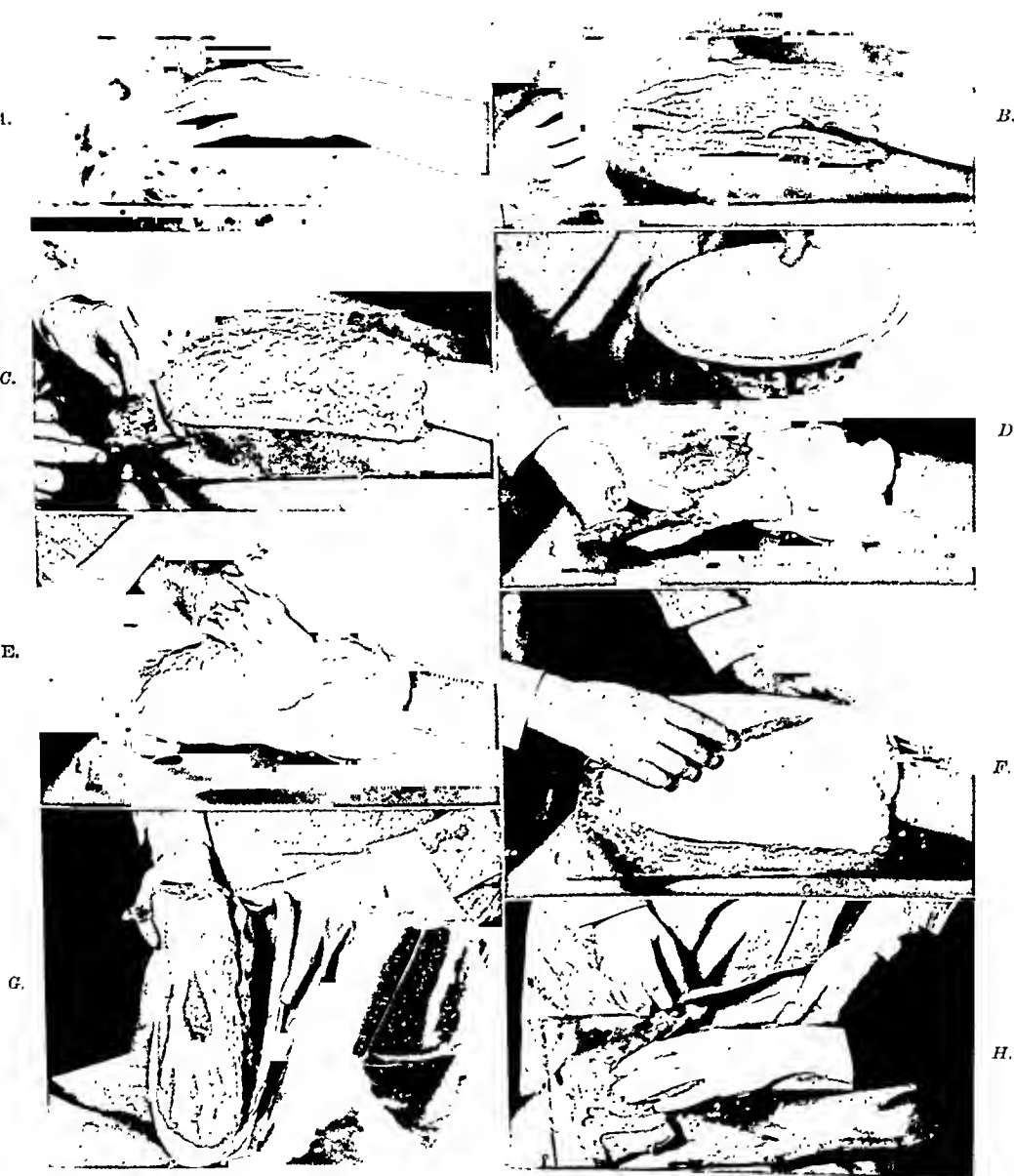


Fig. 3.—The painting rather than pouring method of making an agar mold of an animate object. This method uses less agar and requires about the same amount of time to make.

- A, The hand is placed on a piece of glass.
- B, The agar composition is painted on the glass around the hand to anchor it into position. It is then applied over the hand.
- C, Rough edges of the agar are cut off and scraped away.
- D, Plaster which has been mixed in cold water is painted over the agar mold. If the mold has not completely set, the cold water-plaster mixture hastens setting for the agar mold.
- E, As the plaster thickens in setting, it is applied with the hands until sufficient thickness is built up.
- F, The top of the mold is scraped flat so that when inverted it will be level for pouring the positive.
- G, After the wax positive has been made, the agar mold may be taken from the plaster case.
- H, The agar mold is broken from the cast in removing the positive. This is given a coat of shellac fixative and colored.

quality of the finished product. For example, an impression of the hand may be made by painting or pouring the mixture over the skin surface, as seen in the illustrations. Both methods have their advantages and disadvantages. Invariably, less of the mixture is required in a painting process than in a pouring process.

Thin agar molds are not sufficiently strong by themselves to hold their shape without crumbling or cracking. It has, therefore, become necessary to



Fig. 4.—Making the agar mold of a face. This may be done while the subject is sitting erect. However, a rest for the head should be provided.

A, A small amount of agar is placed on the back of the hand of the subject to test its temperature. If it is too hot, it will not only be uncomfortable, but it must remain on the face longer to allow complete setting.

B, When the temperature is correct, the agar composition is applied from the neck upward. This prevents the agar composition from flowing too rapidly because of the pull of gravity.

C, Care must be taken in working the composition around the nose to allow openings for breathing. In this illustration the composition is allowed to run between the openings of the nostrils.

D, As the work progresses upward, the composition is worked carefully around the openings to the nose. Even if the nostril is covered, normal breathing will cause it to open. The composition will not be sucked into the nose unless it is too fluid.

follow one of two procedures to obtain a good mold. The first is to apply a plaster or a wax-resin shell over the thin agar mold so that it will not warp or break. The other method is to reinforce a thick agar mold with metal supports or with an armature.<sup>3</sup> A mask may be made from copper or brass wire or from hollow tubes. This tubular mask is illustrated and described by Kaufman.<sup>4</sup> The mask made of tubes is superior to the wire one because cold water may be



Fig. 5.—Making the plaster shell to support the agar mold.

A, After the agar mold has completely covered the face, the plaster-water mixture is applied.

B, As the plaster becomes thick during the setting, it is applied with the hand until sufficient thickness is built up.

C, The completed plaster shell. It will be noticed that openings have been left for breathing. Although straws placed in the nostrils could have been used, better results may be obtained if the worker masters the technique of handling both agar and plaster.

D, Agar molds are more easily removed from the face than plaster molds. In fact, the agar mold will generally fall from the face by its own weight when the face is placed parallel with the floor. A separating medium is necessary when making a plaster mold and should be used before the plaster is applied.

<sup>3</sup>A suitable wire mask is sold by the Warren-Knight Company, Philadelphia.

forced through the tubes and these aid considerably in cooling the agar composition to its setting point. However, both the wire and tubular masks have two disadvantages. Seldom do they fit snugly or closely enough to the skin surface to allow a thin mixture of agar to be applied. If they are anchored to the agar mold with gauze ties previously dipped in agar, their efficacy is reduced and much time is consumed in the process. Less time is required to make a thin plaster shell. Although an agar mold may be reinforced with



Fig. 6.—Making an agar mold of a profile.

A, A cardboard cutout is made to cover the profile of the face. This may be done in one of two methods. A piece of soft solder wire is bent over the face to take the outline of the face and head. This is removed and placed on the cardboard, and its inner surface is traced. The space is then cut out of the cardboard with the scissors. The other method is to place the head against a piece of cardboard. A pencil is then held vertical to the cardboard and an outline of the face is traced. These outlines seldom are absolutely accurate. Open spaces often exist between the face and the cardboard. Pieces of gauze are laid over these openings as the agar is applied. The gauze prevents the agar from running through the opening. As the work progresses from the bottom upward, the gauze is moved adjacent to the face. In cases of very loose hair, as seen in this illustration, it is advisable to give the hair a thin coating of petroleum jelly to prevent the agar from flowing too deeply into the hair. However, this can be dispensed with if the agar composition is applied with a syringe or grease gun having a nozzle which can produce ribbons of agar. Similar devices are used for decorating cakes.

B, The rough outline is cut away after the agar mold is completed. The subject can breathe through the nostril that is not covered with agar. If breathing is difficult through one nostril, an opening is left in the opposite side as the agar mold is applied.

C, The plaster shell is applied after the agar mold is completed.

D, The completed shell covering the agar mold.

gauze dipped in a liquid agar composition, such masks are less effective than the simple application of a thin plaster shell because more of the agar composition is required to make a thick mold, and this takes longer to set. The resulting thick agar mold is seldom as strong as the thin agar mold that is reinforced with a thin plaster shell. Furthermore, when one layer of agar composition is applied over a set layer, the different layers will probably separate and produce a weakened, crumbly mold.



Fig. 7.—The wax cast made from the agar mold seen in Fig. 6 ready for coloring.

A single thin painted layer is generally reinforced by means of a plaster rather than a wax-resin shell or case. It would be logical to reason that this double molding process requires more time to complete. On the contrary, this is not true, for the thin layer of agar composition sets quite rapidly and the plaster may be mixed thick, or its setting time may be accelerated by the addition of sodium chloride or potassium alum. It is practical because detail on the inner surface of the case or shell is not necessary. In this manner both the agar mold and the shell set rapidly, in the same length of time that would be required for a thick agar mold or a detail plaster mold to set.

The thin layer of agar composition obtained in painting shrinks and cracks rapidly. Therefore, the cast must be made soon after the mold is removed from the subject or the mold must be kept in wet towels if considerable time elapses between the making of the mold and the making of the cast. The same rule applies to the making of impressions of the face as well as other surfaces of the body. We have learned from experience that in spite of these drawbacks better results are obtained with the thin agar mold and the reinforcing plaster shell.

Here it may be contended that a plaster mold could have been made in the beginning without the use of agar. This is erroneous for three major reasons: (1) The plaster does not record as much detail as the agar; (2) the mold is more difficult to remove from the subject; and (3) the cast is decidedly more difficult to remove from the mold.

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# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

TISSUE, A New Staining Method for Gram-Positive and Gram-Negative Organisms in Frozen Sections, Krajian, A. A. Arch. Path. 32: S25, 1941.

## Method:

1. Prepare frozen sections, 7 to 10 microns thick, in the usual manner.
2. Stain for two minutes in alum-hematoxylin (Harris' method).
3. Wash in tap water until blue, and destain rapidly in acid alcohol, dipping in and out five to seven times.
4. Rinse in tap water and apply copper sulfate-zinc solution for three minutes (7 Gm. of copper sulfate and 4 Gm. of zinc sulfate dissolved in 100 c.c. of distilled water with the aid of heat).
5. Pour off, and apply brilliant green solution for five minutes (0.3 Gm. of brilliant green dissolved in 10 c.c. of copper-zinc sulfate mixture). Rinse in water and fortify for one minute with a 5 per cent aqueous solution of ammonium nitrate.
6. Rinse in tap water and apply carbolfuchsin (Ziehl-Neelsen method) for two minutes.
7. Rinse in tap water, blot, and apply dioxane for two minutes.
8. Pour off, and without washing apply creosote-xylene (equal parts), changing the solution several times and agitating the slide for even differentiation until the background appears to be clear red with no more stain leaving the section. (This step requires about one minute, and it is advisable to control the differentiation under the microscope.)
9. Clear in pure xylene for two minutes.
10. Mount in gum dammar.

With the use of this method, nuclei stain bluish red; gram-positive organisms, bluish green; gram-negative organisms, red; monillias and actinomycetes, green; and Negri bodies, bright red with greenish chromatin bodies.

All the staining solutions are stable, except the brilliant green, which keeps well for about twenty-four hours.

UROBILINOGEN, Determination of, in Feces and Urine. Watson, C. J., and Bilden, E. Arch. Int. Med. 65: 740, 1941.

Direct comparison has been made of the Sparkman and the Watson procedure for estimation of urobilinogen in feces and in urine.

Sparkman's report of higher values obtained with his technique, particularly as applied to urine, has not been confirmed. Opposite result were noted in the present investigation. Reasons for this discrepancy are discussed.

Evidence is presented which indicates the desirability of (a) obtaining an average per diem value for excreted urobilinogen, especially for fecal urobilinogen, over a period of several days, and (b) using a method which permits concentration of small amounts of urobilinogen whenever necessary because of low concentration in the sample.

B. DYSENTERIAE (Flexner). A Tellurite Rosolic Acid Medium Selective for, Wilson, W. J., and Blair, E. M. McV. Brit. M. J. Oct. 11, p. 501, 1941.

To 100 c.c. of melted nutrient agar cooled to 60° C. in a flask are added 0.5 c.c. of 1 per cent rosolic acid dissolved in absolute alcohol, 1 c.c. of a 4 per cent watery solution of iron alum, and finally 3 c.c. of a lactose-tellurite solution. The latter solution is made by boiling 20 Gm. of lactose in 100 c.c. of distilled water, cooling the solution, and then

dissolving in it 0.2 Gm. of potassium tellurite. It is necessary to dissolve the tellurite in a cold temperature, otherwise reduction occurs. This lactose-tellurite solution keeps for weeks, the tellurite being an effective preservative against bacteria, but not against molds. Growth of the latter can be prevented by the addition of 3 per cent of ether and by keeping the bottle tightly stoppered.

When the contents of the flask containing the medium have been thoroughly mixed, they are poured out into Petri dishes and allowed to set with the lids removed. A large loopful of a thick fecal emulsion in peptone water is spread over the surface of the plate and allowed to dry in the air. After incubation at 37° C. for eighteen hours, the pink colonies of Flexner's bacillus are readily distinguished from any yellow colonies of resistant *B. coli* which may have developed. Flexner's bacillus forms alkali, and the colony becomes distinctly pinkish red. In most cases the growth can be tested at once on a slide for agglutination with a polyvalent Flexner agglutinating serum.

**PROTHROMBIN DEFICIENCY in Pulmonary Tuberculosis, Sheely, R. F. J. A. M. A. 117: 1603, 1941.**

Among 106 cases of active and chronic pulmonary tuberculosis (10.3 per cent of minimal, 20.6 per cent of moderately-advanced, and 69.1 per cent of far-advanced tuberculosis) studied in regard to the concentration of prothrombin in the blood, a significant deficiency of prothrombin was found in 51.

Fifty patients had far-advanced tuberculosis, and 5 of these were receiving treatment with artificial pneumothorax, with resulting unsatisfactory collapse of the diseased lung. Two patients were undergoing re-expansion of the collapsed lung.

Among the patients, 73 per cent with minimal, 60 per cent with moderately-advanced, and 9.6 per cent with far-advanced tuberculosis had a prothrombin concentration within the normal range. (A total of 68.5 per cent of those with far-advanced tuberculosis had a prothrombin concentration below 59 per cent of normal.)

There was no constant relation between the prothrombin concentration and the sedimentation rate. The status as to prothrombin level appeared to coincide with the clinical and roentgenologic status of the majority of the patients.

It is suggested that the prothrombin concentration should be determined in every case of hemoptysis in pulmonary tuberculosis. In 4 cases of frank hemoptysis with associated prothrombin deficiency, the elevation of the blood prothrombin level on the administration of vitamin K, orally and parenterally, was a likely factor in the control of the hemoptysis.

The significance of the prothrombin concentration in surgical procedures on patients with pulmonary tuberculosis must await further study.

The prothrombin concentration in the blood of patients with tuberculosis seems to be related, in some way, to the toxemia resulting from the existing tuberculous infection in the lung.

**SULFANILAMIDE, A Simplified Method for the Estimation of Sulfapyridine and, in Biological Fluids, Scott, L. D. Brit. J. Exper. Path. 22: 220, 1941.**

1. (a) Stock standard of M & B 693 Dagenan. One hundred milligrams of pure 693 dissolved in 100 c.c. of N/100 sodium hydroxide. A special preparation of 693 is supplied for chemical analysis by May and Baker, Ltd., Dagenham, England.

(b) Stock standard of sulfanilamide, 100 mg. of paraaminobenzene-sulfonamide (B.D.H.) per 100 c.c. of water.

2. Standard solutions of 693 and sulfanilamide. Two standards are prepared of each, 2.5 and 5 c.c. being diluted to 100 c.c. with water. One cubic centimeter equals 0.025 and 0.05 mg. of 693 and sulfanilamide, respectively.

3. Three per cent (by volume) hydrochloric acid.

4. Dilution 1:10 with water of 0.5 per cent aqueous sodium nitrite solution. (The 1:10 dilution is prepared prior to the test or freshly each day.)

5. Fifty per cent aqueous solution of urea.



6. One per cent (by volume) alcoholic solution of dimethyl- $\alpha$ -naphthylamine prepared freshly each month from stock solution (B.D.H.), and stored in an amber ground-glass stoppered bottle.

7. Diluent for colorimetry: 20 c.c. of water, 5 c.c. of 3 per cent hydrochloric acid, and 5 c.c. of absolute alcohol.

8. Normal horse serum and oxalated whole blood (Burroughs Wellcome & Co.).

9. Approximately 0.25 per cent protein solution prepared by a 1:25 dilution of the horse serum with 0.5 per cent sodium chloride. Five to 6 drops of 10 per cent alcoholic thymol solution is added as preservative.

10. Cordite tubes of uniform bore, graduated in 0.1 c.c. and of 5 c.c. capacity (diameter 1 cm., length 12.5 cm.).

*Note.* Solutions 1, 2, 6, 8, 9 if kept in the refrigerator are stable indefinitely.

*Procedure for Cerebrospinal Fluid.*—Introduce into two test tubes (graduated at 5 c.c.) in the following order, with thorough mixing after each addition:

| <i>Standard</i>                            | <i>Test Cerebrospinal Fluid</i>             |
|--|---|
| 0.5 c.c. of standard (0.05 mg. per c.c.)   | 1 c.c. of cerebrospinal fluid               |
| 0.5 c.c. of 0.25 per cent protein solution | 0.5 c.c. of 3 per cent hydrochloric acid    |
| 0.5 c.c. of 3 per cent hydrochloric acid   |   |
| 0.5 c.c. of 1:10 dilution of sodium        | 0.5 c.c. of 1:10 dilution of sodium nitrite |

Stand at room temperature for five minutes.

Add 0.5 c.c. of 50 per cent urea solution to each tube, mix well, and allow standing for three to five minutes longer for the urea to destroy excess of sodium nitrite. (The unused residual nitrous acid otherwise reacts with the dimethyl- $\alpha$ -naphthylamine to form a brownish-orange coloration that makes colorimetric comparison somewhat difficult.)

Add 0.5 c.c. of 1 per cent alcoholic dimethyl- $\alpha$ -naphthylamine to the contents of each tube, and mix very thoroughly. Allow to stand for ten minutes, and then dilute both volumes to the 5 c.c. mark with the special diluent fluid. Mix again, and compare colorimetrically or by dilution colorimetry.

Calculation:  $\frac{S}{U} \times 0.025 \text{ mg.} \times 100 =$  milligrams of sulfonamide  
per 100 c.c. of cerebrospinal fluid.

*Procedure for Blood Serum.*—

| <i>Standard</i>                                   | <i>Test Serum</i>                           |
|---|---|
| 0.5 c.c. of normal horse serum                    | 0.5 c.c. of serum                           |
| 0.5 c.c. of standard (0.025 or 0.05 mg. per c.c.) | 0.5 c.c. of water                           |
| 0.5 c.c. of 3 per cent hydrochloric acid          | 0.5 c.c. of 3 per cent hydrochloric acid    |
| 0.5 c.c. of 1:10 dilution of sodium nitrite       | 0.5 c.c. of 1:10 dilution of sodium nitrite |

Stand at room temperature for five minutes.

*Note.*—It should be noted that the occurrence of hemolysis renders this technique useless, it being impossible to match the colors of standard and test solution.

Add 0.5 c.c. of 50 per cent urea solution to each tube, stand again for three to five minutes, and then add 0.5 c.c. of dimethyl- $\alpha$ -naphthylamine to the contents of both tubes. Mix and allow to stand for ten minutes, after which the volumes are diluted to 5 c.c. with the dilution fluid. Mix thoroughly and compare in the colorimeter or by colorimetry.

Calculation:  $\frac{S}{U} \times 0.0125 \text{ or } 0.025 \text{ mg.} \times 200 =$  milligrams of sulfonamide per 100 c.c. of serum.

*Procedure for Whole Blood and Lipemic or Hemolyzed Sera.*—

Into a small test tube introduce 2 c.c. of water, and add 1 c.c. of whole blood, serum, or plasma. After mixing well, stand for two to three minutes for plasmolysis to take place. Add 2 c.c. of 15 per cent trichloroacetic acid, drop by drop, with constant shaking. Finally shake the mixture vigorously and filter it through a No. 1 5.5 cm. Whatman paper. Pipette

2.5 c.c. of filtrate into a 5 c.c. graduated test tube, and add 0.5 c.c. of 1:10 dilution of sodium nitrite. Allow the tube to stand for five minutes at room temperature and then add 0.5 c.c. of urea solution, followed by 0.5 c.c. of alcoholic dimethyl- $\alpha$ -naphthylamine.

The standard is prepared at the same time by adding 0.25 c.c. (with a micropipette) of stock sulfonamide solution (100 mg. per 100 c.c.) to 4.75 c.c. of oxalated horse blood or horse serum as the case may be. Mix thoroughly and pipette 1 c.c. into 2 c.c. of water. The above procedure for the test blood is then followed in detail.

After the addition of the coupling reagent to the standard and test solutions, the colors are allowed to develop at room temperature for fifteen minutes, and are then diluted to 5 c.c. with the following diluent: 25 c.c. of water, 10 c.c. of 15 per cent trichloroacetic acid, and 5 c.c. of absolute alcohol. The contents of each tube are mixed well and comparison is made by dilution colorimetry (using the diluent just described), or by colorimetric means.

$$\text{Calculation: } \frac{S}{U} \times 5 = \text{milligrams of sulfonamide per 100 c.c.}$$

*Estimation in Urine.*—A 1:5 dilution or a 1:10 dilution of urine is made with the 0.25 per cent protein solution, and 0.5 c.c. is taken and compared with a suitable standard.

| <i>Standard</i>   | <i>Urine</i>   |
|---|--|
| 0.5 c.c. of standard (0.025 or 0.050 mg. per c.c.)  | 0.5 c.c. of 1:5 or 1:10 dilution of test urine with 0.25 per cent protein solution |
| 0.5 c.c. of normal control urine diluted in the same proportion as the test urine with 0.25 per cent protein solution | 0.5 c.c. of water  |

Proceed as for the blood serum method.

$$\text{Calculation: } \frac{S}{U} \times 0.0125 \text{ or } 0.025 \text{ mg.} \times \text{dilution of urine} \times 200 = \text{milligrams of sulfonamide per 100 c.c. of urine.}$$

With other body fluids, such as transudates or exudates, treat exactly as for the blood serum method.

## REVIEWS

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Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughnau, Professional Building, Richmond, Va.

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### The Advancing Front of Medicine\*

AN EXCELLENT volume dealing with recent advances in all fields of medicine, prepared for the intelligent layman. The various chapters are elaborations of short articles which have recently appeared in *Harper's Magazine* and elsewhere. By virtue of his many years of connection with The Rockefeller Foundation, Dr. Gray has developed a facility in understanding and presenting, in a delightful manner, information in science and in medicine. Physicians will enjoy this book as much as laymen. It is up to the minute and completely authentic.

### Stethoscopic Heart Records†

THIS is a book only in the sense that it is a bound volume. The binding covers a series of seven Columbia single-faced records, prepared by Dr. Geckeler for student instruction in cardiac auscultation. The records are played on the phonograph. The auditor sits wearing his stethoscope and the sounds appear to come through the stethoscope, simulating in a remarkable manner what one actually hears at the bedside. There is a running commentary describing what will be heard next.

Normal heart sounds, abnormal heart sounds, murmurs, and arrhythmias are illustrated.

The reviewer was impressed with this excellent idea for student instruction. It will probably also find value among those medical graduates who like to check from time to time on the accuracy of their work. It might be described as a very short refresher course.

One very minor criticism is that at one point the commentator may be misunderstood as implying that one can tell the difference between syphilitic and rheumatic aortic valve diseases by the character of the murmur.

### Diseases of the Blood‡

THE book is just what it purports to be, an atlas of hematology. It is beautifully illustrated with many full-page color plates. The text covers not only the pathologic findings but also the diagnosis and treatment of the various blood dyscrasias. An example of the thoroughness of coverage is found in Table XIX, which lists the relative cost to the patient per unit of various brands of liver extract.

Hematology has developed a language of its own, and the first two chapters deal with terminology and definitions of hematologic terms. There follows a discussion of the origin and development of blood cells, and a long section presenting what is known concerning the normal blood and marrow constituents. The major portion of the book is given over to blood diseases.

The volume should be of great value to clinical pathologists and internists.

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\*The Advancing Front of Medicine. By George W. Gray. Cloth, 425 pages, \$3.00. McGraw-Hill Book Company, Inc., New York and London, 1941.

†Stethoscopic Heart Records. Sounds, Murmurs and Arrhythmias. By George D. Geckeler, M.D., Columbia Records, Set M600, 1941.

‡Diseases of the Blood and Atlas of Hematology. With Clinical and Hematologic Descriptions of the Blood Diseases, including a Section on Technique and Terminology. By Roy R. Kracke, M.D., Professor of Bacteriology, Pathology, and Laboratory Diagnosis, Emory University School of Medicine; Pathologist to the Emory University Hospital; Consultant in Hematology to the Grady Hospital and Eggleston Hospital for Children, Atlanta, Ga. Former ed. 2, thoroughly revised, reset and enlarged. 692 pages, 54 color plates and 46 other illustrations, \$15.00. J. B. Lippincott Company, Philadelphia, London, Montreal, 1941.

## Immunology\*

ONE is surprised at the paucity of recent books written primarily for discussion of immunology. Most such volumes cover bacteriology and immunology, with major emphasis on the former. Sherwood's book, therefore, stands alone. If there were a profusion of other books, it would still stand alone. This is probably the chief reason why the second edition appeared soon after the first. New chapters have been added, a few changes suggested by reviewers of the first edition have been made, and the subject has been brought up to date.

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## The Retina†

COVERING much more than its title implies, *The Retina* is a most comprehensive review of anatomy of the retina and the visual pathways and the physiology of vision. The tremendous bibliography illustrates well the thoroughness with which the author has covered his subject in his ten years of investigation. Much of the contents represents original study. There are one hundred full-page plates.

This volume should be of especial interest to ophthalmologists, neurologists, and physiologists. For others it will serve more as a reference volume.

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## Synopsis of Allergy‡

THE *Synopsis of Allergy* is an excellent addition to the Mosby Synopsis Series. Dr. Alexander has an admirable facility for compressing much knowledge into little space. To accomplish this he has necessarily omitted discussion of pros and cons of moot points. The omission of extensive references to the contributions of others makes for easier reading and saves much space. These references being available in other larger works on allergy, there is no need for their inclusion in a small book the prime function of which is to give meat.

A few sections, especially those on serum allergy, bacterial allergy, and drug allergy could be elaborated upon with no great increase in size of the volume.

A short appendix gives the desired basic information concerning preparation of extracts and methods of diagnostic study in such a way that one who has made no special study of allergy could carry on with the diagnosis and treatment of simple cases. More complicated cases would require reference to more extensive treatises on the subject.

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## Allergy in Clinical Practice§

ANOTHER addition to the profusion of recent books on allergy; this volume makes no effort to add anything basically new to the subject, and is presented with the same general type of approach that is followed in *Medical Clinics of North America*. It is primarily a series of case reports from the Cleveland Clinic, designed to illustrate the advantage of collaborative work with the allergist on the part of specialists in the several other fields of medicine. The outstanding sections are those on Allergic Dermatoses and Ocular Manifestations of Allergy. References to the literature are rather scant.

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\*Immunology. By Noble P. Sherwood, Ph.D., M.D., F.A.C.P., Professor of Bacteriology, University of Kansas, and Pathologist to the Lawrence Memorial Hospital, Lawrence, Kan. Cloth, ed. 2, 639 pages, illustrated, \$6.50. The C. V. Mosby Company, St. Louis, Mo., 1941.

†The Retina. The Anatomy and the Histology of the Retina in Man, Ape, and Monkey, Including the Consideration of Visual Functions, the History of Physiological Optics, and the Histological Laboratory Technique. By S. L. Polyak, M.D., A Fiftieth Anniversary Publication of the University of Chicago Press. Cloth, 607 pages, \$10.00. The University of Chicago Press, Chicago, Ill., 1941.

‡Synopsis of Allergy. By Harry L. Alexander, A.B., M.D., Professor of Clinical Medicine, Washington University School of Medicine, St. Louis; Editor of The Journal of Allergy. Cloth, 246 pages, illustrated, \$3.00. The C. V. Mosby Company, St. Louis, Mo., 1941.

§Allergy in Clinical Practice. By Staff-Members of the Cleveland Clinic under the direction of Russell L. Haden, M.D., F.A.C.P., Chief of the Medical Division. Edited by J. Warlick Thomas, M.D., F.A.C.P., Chief of the Section on Allergy. Cloth, 354 pages, 92 illustrations, including 14 in color. J. B. Lippincott Company, Philadelphia, Montreal, London, 1941.

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## CLINICAL AND EXPERIMENTAL

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### THE ORIGIN AND NATURE OF THE CABOT RING BODIES OF ERYTHROCYTES\*

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EMIL MARO SCHLEICHER, MINNEAPOLIS, MINN.

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#### INTRODUCTION

CABOT<sup>1</sup> in 1903 enriched the literature with an unique contribution. He described red and blue staining circular and twisted bodies within basophilie, polychromatie, and infrequent orthochromatie erythrocytes, first brought to his attention by one of his associates in a case of lead poisoning and thereafter observed by himself in the anemic blood of patients suffering from pernicious anemia, lead poisoning, and lymphatic leucemia.

The dissertation aroused much interest for a twofold reason: first, no previous mention of such bodies had been made; and second, Cabot's hypothesis that the ring bodies may represent "nuclear remnants" was diametrically opposed to the tinctorial behavior of the structures.

When this paper went to press, the literature contained no feasible explanation for the peculiar affinity of these supposed nuclear derivatives for cytoplasmic stains; no actual proof withstanding critical analysis that the ring bodies are nuclear remnants, and no convincing data to show that they are laboratory creations.

It is the purpose of this communication to show that the Cabot ring bodies are denatured protein configurations, i.e., they are actually laboratory products. The phenomenon occurs in specifically altered cells only, i.e., in erythrocytes and certain erythroblasts that have undergone lysis. This statement is supported by two facts: (a) the creation of numerous Cabot ring bodies at will, in blood samples taken from cases with pernicious anemia in severe relapse, acute lead

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Received for publication, May 17, 1941.

poisoning, terminal lymphosarcoma, acute malaria, terminal lymphatic leucemia, congenital hemolytic icterus during hemolytic crisis, erythroblastosis fetalis, and a case with generalized Hodgkin's disease; and (b) the production of simulacra in normal, pathologic human erythrocytes, and the creation of analogous structures with egg white by special methods.

For those who are not familiar with the problem at hand the major articles and theories will be presented briefly. Cabot's dictum that divided students of the subject into two groups, those who advocate that the ring bodies are remnants of the nuclear membrane, and those who claim that they are artifacts, is cited first:

"There is no reason to believe them a mark of cellular degeneration . . . they have some connection with the nucleus formerly possessed by the cell that contains them seems to me not unlikely . . . their shapes correspond for the most part very well with those of nuclear figures . . . the best hypothesis, therefore, that I can suggest to account for these appearances is that they represent nuclear remains . . . the significance of their red color with Wright's, a stain which never stains any nucleus red, is not clear to me . . . have these figures any practical significance? Not so far as I know."

Thus Cabot, while very conservative and noncommittal regarding the nature of the bodies, precipitated, by this very approach to the phenomenon, an extensive literature dealing with the mode of origin of the structures bearing his name.

Among the investigators who believe the rings to be identical with the nuclear membrane are Schleip,<sup>2-3</sup> Pappenheim,<sup>4-6</sup> Sluka,<sup>7</sup> Alder,<sup>8</sup> Ferrata,<sup>9,10</sup> Ferrata and Viglioli,<sup>11</sup> Jnsa,<sup>12</sup> and Jordan, Kindred, and Beams.<sup>13</sup> Ferrata's hypothesis: "The Cabot ring bodies are the peripheral residue of the nucleus and are produced by vacuolization of the central mass" has become a prevailing view, in spite of his observation that generally the rings stained with pyronin and only an occasional one showed affinity for methyl green, specific for nuclear derivatives. In Ferrata and Viglioli's paper, the concept was enlarged by the authors' statement that "the Cabot ring bodies are morphologically identical with the peripheral part of the nuclear membrane. They are always a sign of a typical pathological maturation of the erythroblasts, the ring bodies occur only in severe anemias." Jnsa proposed the following theory: "The ring bodies are not typical nuclear chromatin; they represent nuclear remnants and in all probability are the membrane left behind through pathological endonuclear vacuolization processes and karyolysis." Jordan and co-workers concluded: "Moreover, the genuine Cabot rings seem clearly to represent persistent nuclear membranes."

Among those who proposed that the Cabot rings are artifacts is Demel,<sup>14</sup> who assumed that "the Cabot rings represent the boundary zone between an inner area of more rarefied hemoglobin and an outer more condensed area." Demel was working with normal blood, using a stain (Neo-Giemsa) formulated by Dr. Poletti of Pisa. His theory is refuted, however, by the fact that the rings can be produced after the hemoglobin has been laked.

Russow,<sup>15</sup> commenting on Sluka's paper, pointed out that if the Cabot rings are nuclear remnants, they should be seen in the bone marrow, and their appear-

ance should obviously be more frequent in this organ. He concluded: "the rings are most likely the expression of unusual drying processes of the anemic blood, since the bodies have only been observed in dried peripheral blood preparations."

The neutral side in the controversy is represented by Naegeli,<sup>16</sup> who summarized the literature as follows: "Cabot rings are generally considered nuclear membrane remnants, absent in erythroblasts and the blood forming organs; they are a pathologic phenomenon of nuclear dissolution." Schilling<sup>17</sup> states: "It is not certain that the Cabot-Schleip rings are nuclear remnants." In another publication<sup>18</sup> he records seeing Cabot rings in normal erythroblasts. He further suggests, according to Schröder,<sup>19</sup> that the rings may represent the contour of the "glass body" of the erythrocyte. Isaacs,<sup>20</sup> in Downey's *Handbook of Hematology*, informs us that Cabot rings have been found in cells with perfectly intact nuclei and entirely separate from the nuclei. Isamboulas and Malikiosis<sup>21</sup> present in their article a photomicrograph showing a Cabot ring in the protoplasm of a normal erythroblast obtained by sternal aspiration from a patient with typhus. They come to the conclusion that their observation is "opposed to the prevailing view."

That Cabot rings occur in erythroblasts has been ascertained from the literature cited and from my own observations, but their presence is rare indeed. Over a period of many years I have seen only two specimens. The infrequency is especially brought out by the fact that no clear-cut example was noted by Dr. Hal Downey, in his large collection of preparations in the field of hemopathology. The erythroblast shown in Fig. 14 was found in one of the smears made from the peripheral blood of the case with generalized Hodgkin's disease mentioned above. The only other one was seen in the bone marrow of a patient with severe relapsed pernicious anemia, having about 650,000 red blood cells per c.mm. A probable reason for the scarcity of the presence of Cabot rings in erythroblasts is given elsewhere in this paper.

In over 2,150 bone marrow aspirations,<sup>22, 23</sup> covering practically all well-known hematologic disorders (mindful of the admixture of sinusoidal blood with the bone marrow specimen), I have in no instance observed a greater number of Cabot rings in the bone marrow preparations than were seen in those made from the peripheral blood. No exception was the case with acute sickle-cell anemia,<sup>24</sup> where in the "usable area"<sup>25</sup> the erythroblasts numbered eighty-four among one hundred leucocytes, but only four erythrocytes with Cabot rings were observed per 180 micrometer squares.<sup>26</sup> The scarcity of Cabot ring bodies seemingly is the rule rather than the exception in severe erythroblastic anemias and toxic anemias associated with erythroblastemia and icterus (leading by their very nature to severe disturbances of the denueation process), where one would expect Cabot ring bodies to be plentiful in the bone marrow. As already pointed out, this is not the case. Furthermore, dark-field examinations have never revealed that Cabot ring bodies are preformed structures, i.e., nuclear membranes. The hypothesis that the Cabot ring bodies are neither nuclear remnants nor a sign of pathologic denueation processes, is strengthened by the observations cited in this paragraph.

Structures similar to those described by Cabot have been produced by various investigators.<sup>27, 28, 38, 45-47</sup> These simulacra were obtained by exposing erythrocytes to a hemolytic system. It may then be assumed that the creation of the ring bodies depends upon the degree of lysis an erythrocyte or erythroblast has undergone in vivo or in vitro, induced by a specific agent. Thus a plausible reason is advanced for the appearance of these bodies in disorders of the hematopoietic organs associated with severe anemias, and with or without elevated icterus.

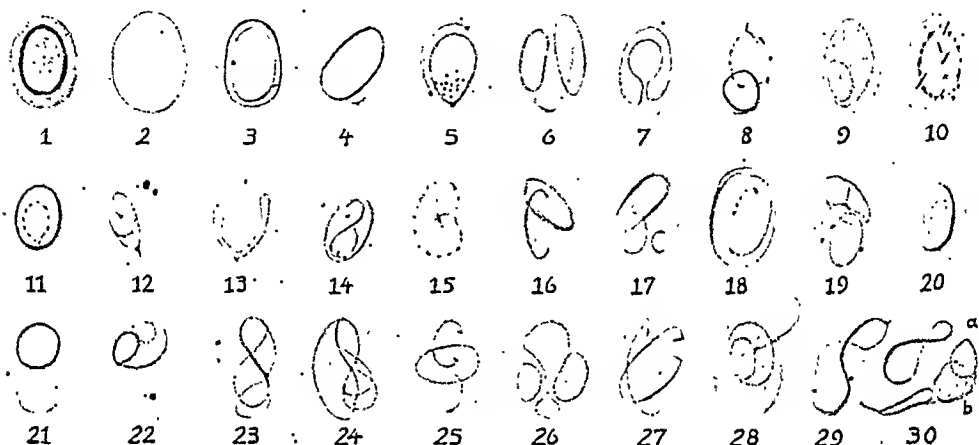


Plate I.  
Basophilic and polychromatic erythrocytes showing "denatured protein configurations" (Cabot ring bodies).

#### THE ORIGIN OF CABOT RING BODIES

A blood smear was presented to me by Dr. R. H. Jaffé\* in which he had observed Cabot ring bodies showing an unusually brilliant red hue, as well as width and flatness of the ring bodies. The smear was made from the peripheral blood of a moribund patient with generalized Hodgkin's disease. Figs. 1 and 2a show examples of the bodies seen by Dr. Jaffé. Examination of the preparation revealed that there were only three erythrocytes with Cabot ring bodies per 180 micrometer squares.<sup>26</sup> Erythroblasts were numerous in this blood, and many cells showed pathologic denucleation. A severe anemic state was present: red blood cells, 1,020,000, hemoglobin, 3.0 Gm., icterus index, 15 (Wintrobe), and a reticulocytosis of 18.4 per cent; occasionally a reticulocyte with ring bodies, Figs. 2 to 6. As the number of ring bodies was too small to trace their origin, peripheral blood was defibrinated and permitted to sediment in a Wintrobe hematocrit tube. The procedure was designed to gather more specifically altered red cells assuming that hemolyzed cells tend to form the uppermost layer of the blood column. While smears made from the top layer yielded a larger number of ring bodies, I was unable to trace intermediate stages between the nuclear membrane and the Cabot ring bodies.

\*Chief pathologist of Cook County Hospital, Chicago, Ill.



An experiment was then set up that conformed to Russow's theory. Peripheral blood was spread on glass slides, placed in a slanting position while drying, allowing the plasma to accumulate near one end of the blood films, and thus delaying the drying process. The control slides were made in the standard manner, rapidly whipping them through the air to accelerate drying. On examination of the preparations, the following observations were made: Many circular and twisted Cabot ring bodies were seen in the area where drying was delayed, whereas the opposite area showed only occasional bodies. The control slides revealed about the same number of ring structures as noted in the rapidly drying area of the slanting preparations. This was definite evidence that the ring bodies were created by delaying the drying process. Plate I shows some of the Cabot ring bodies produced by the slanting method.

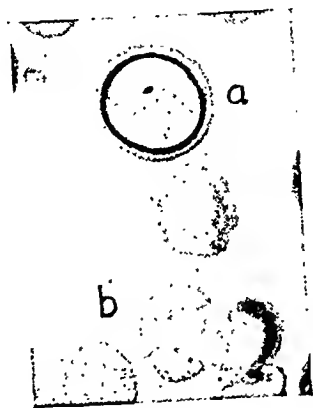


Fig. 1.

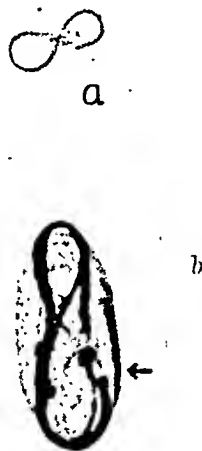


Fig. 2.

Fig. 1.—Mr. A. K. Generalized Hodgkin's disease, peripheral blood. *a*, Orthoehromatic erythrocyte with a typical Cabot ring body. Note that this denatured protein body has a considerable width and appears to be a flat structure within a uniformly stained cell. *b*, Markedly lysed erythrocyte with a fine blue ring.

Fig. 2.—Mr. A. K., peripheral blood. *a*, Erythrocyte with a figure-eight body and a bar at the crossing point. *b*, Erythrocyte with a figure-eight body intersecting a figure-eight body at the cell periphery (at arrow).

The formation of the figure eight may be as follows: two rings form simultaneously in the same plane and flow together, or rings unite from adjacent planes, or a ring retracts rapidly and overlaps.

At no time were the angles great enough to encourage concentrations of red blood cells which might account for the increased number of ring bodies at or near the edge of the blood smear. An estimation of the erythrocyte number with the dry chamber method<sup>26</sup> showed an error of from 4.2 to 5.4 per cent for the preparations made by the slanting method, in comparison with the counting chamber number. While in this particular blood the number of ring bodies could be varied at will, this could not be accomplished to the same extent in all blood samples taken from cases with severe anemias, showing one to several ring structures per 180 micrometer squares. In this test series the serum icterus ranged from 2 to 280. When no ring bodies were seen in the routine smears,

their creation by the slanting method frequently met with failure. Fig. 3 shows the development of a Cabot ring body as noted in the areas in which slow drying occurred.

The experiments showed that a severe anemia, high bile pigment concentration, and unusual drying of the blood are not the sole factors responsible for the creation of the phenomenon. The negative results suggested that the formation of ring bodies is dependent upon the presence of a circulating specific hemolysin which alters the relationship of the essential components of the protoplasm while *in vivo*, and that delayed drying, fixing, and staining of the blood are of lesser importance, but are contributory factors. That specific lysis may very likely be a bile derivative "bile acid" that disassociates the chemical components of certain erythroid cells not only *in vivo* but seemingly also *in vitro* is suggested by analytical data presented by Irvin, Johnston, and Sharp\* on bile acids and their hemolyzing effect on the red blood cells *in vitro*.

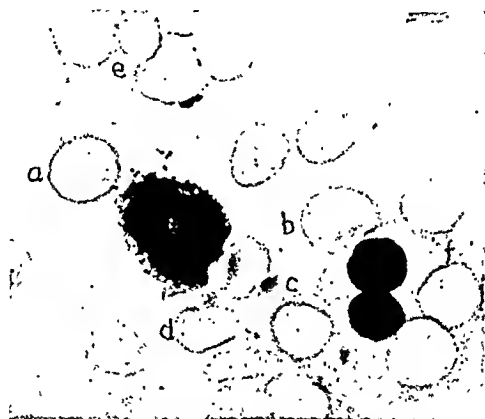


Fig. 3.—Mr. A. K. Peripheral blood showing the development of a Cabot ring body. *a*, Markedly lysed erythrocyte with a reddish corona. Phase I. *b*, Erythrocyte with a pale peripheral area which follows the contour of the cell (for its replica see Plate I, 1). This phenomenon is produced by the retraction of the cytoplasm exposing the transparent envelope. Phase II. *c*, Erythrocyte with retracted peripheral ring. The cell was markedly lysed. The structure had the characteristic red color of Cabot ring bodies. Phase III. *d*, Polkilocyte with irregular aggregated protein at the periphery. See Fig. 27 in Cabot's article. *e*, Group of erythrocytes showing lysis corona approximating the diameter of the ring cell *c*, showing that Cabot's assumption that the bodies may represent nuclear remnants is feasible.

#### STRUCTURE AND TINCTORIAL BEHAVIOR OF THE CABOT RING BODIES

Experiments were designed to determine the reason for the fluctuation of the dimensions, configurations, and affinity to specific cytoplasmic dyes of the ring bodies.

Preparations were used which were made by the slanting method, showing from 12 to 34 erythrocytes with ring bodies per 180 micrometer squares. The location of all cells chosen for this critical study was determined with the vernier scale of a mechanical stage.

Photographs before and after decolorization revealed that some of the ring bodies were at or near the surface, and seemingly part of the envelope, whereas other rings seemed to belong to a layer or layers distinctly below the erythrocyte

\*Annual meeting of the Central Society for Clinical Research, Chicago, Ill., Nov. 1, 1940.

envelope. With the May-Grünwald Giemsa stain these ring bodies were either of a dubious reddish blue, or distinctly red or blue. The ring bodies assumed to be at or near the surface could be made out only with difficulty when decolorization was carried to a point where the nucleus of the leucocytes could still be made out. The deeper lying rings either retained their hue, were a nuance lighter, or became more distinctly red or blue. Some rings which showed irregular staining affinities were invariably composed of irregular thick bars (threads) or of tiny globules. Other ring bodies were composed of not only one, but of two or more thin rings either superimposed on each other or concentrically arranged.



Fig. 4.—Mr. O. H. Pernicious anemia in severe relapse; peripheral blood. *a*. Basophilic megalocyte with a colorless protein ring. The ring, which had a reddish blue tint, could not be retained after the preparation was exposed to room temperature for one week.

Thus it may be assumed that the staining variability depends upon the character and quantity of the substance that makes up a ring, the molecular thickness of such a structure, and the momentary physical state of ring constituents. The latter may supply the answer to the loss of affinity for a dye of a ring body that formerly stained reddish blue. This phenomenon occurred in several basophilic megalocytes from a patient with pernicious anemia in severe relapse when a preparation was decolorized and restained after exposure to room temperature for six days. Fig. 4 shows an example of a colorless ring body in a basophilic megalocyte.

#### STRUCTURE OF THE MATURE ERYTHROCYTE

It is not within the scope of this article to give an account of the numerous hypotheses that have been advanced concerning the structural make-up of the mature erythrocyte. The literature on this particular subject is more confusing than enlightening. The contributions of Michels,<sup>29</sup> Ponder,<sup>30</sup> and others<sup>6, 17, 31-34</sup> give a general picture of this topic.

I have long favored the trend initiated by Ponder to divide the physicochemical structure of the erythrocyte into three components: (a) membrane, i.e., envelope, (b) cytoplasm, and (c) their respective protein-lipoid constituents. This division is not only unreservedly subscribed to by most investigators, but it permits projection of the laboratory experiments presented in this paper into the erythrocytes. All accumulated physicochemical data represent at best only relative values, since the constituents vary with the physiologic state of the subject. The protein component which, as shown elsewhere in this paper, is responsible for the formation of a ring body, constitutes less than 8 per cent of the cell.

Jorpes<sup>35</sup> refers to the protein proper as stroma protein and suggests that it has a characteristic composition different from other proteins of the blood. Its chief properties are (a) consistency, (b) elasticity, and (c) contractility.

Bechhold,<sup>36</sup> while studying the colloidal structure of the erythrocyte, observed the formation of "globules" in normal mammalian red blood cells during the process known as hemolysis. Salén,<sup>37, 38</sup> following up Bechhold's work, used human erythrocytes, and noted with the aid of an ultramicroscope the formation of a ring structure within the cell while it was subjected to a hypotonic saline solution and an acid. The ring was formed at or near the periphery of the cell, and as hemolysis progressed, the ring began to retract, finally degenerating into a refractile granule which eventually disappeared.



Fig. 5.—Normal human erythrocytes treated with Salén's method. *a*, Erythrocyte showing peripheral ring. *b*, Erythrocyte with two rings that originated at different planes forming a pseudofigure eight. *c*, Erythrocyte with disintegrating ring. Note the hook formation. *d*, Erythrocyte with bar and two globules. *e*, Erythrocyte with globules. *f*, Lysed erythrocyte (ghost cell).

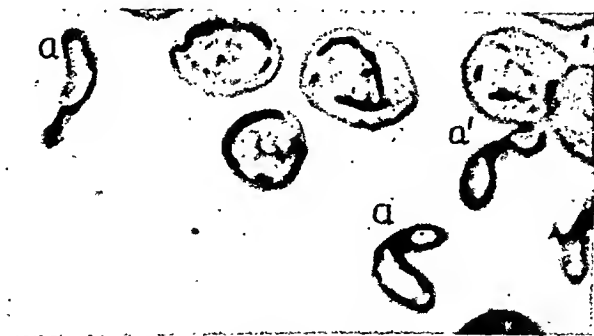


Fig. 6.—Normal human blood treated with Salén's method. *a*, Free protein rings. *a'*, Ring twisted into a figure eight.

Salén's observations were confirmed. Fig. 5 shows an erythrocyte with a peripheral ring, others with stab bodies, some with granules, and one cell without structures. Fig. 6 shows free rings obtained by mechanically rupturing erythrocytes.

Salén reached the following conclusion: "the ring structure seemingly consists of water-insoluble proteins which are precipitated by potassium ferrocyanide dissolved in dilute acetic acid." He thus furnished tangible evidence that a ring structure is formed when hemolysis sets in, thus associating the phenomenon with degeneration of protein.

After correlating the dye analytical studies with Salén's observations, it could be assumed that Cabot ring bodies might be identical with Salén's ring structures, especially since these bodies showed a selective affinity for cytoplasmic stains.

Thus, aggregated and denatured protein forms the Cabot ring bodies.

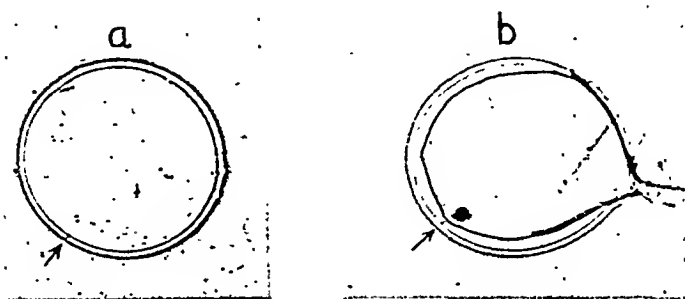


Fig. 7.—Protein globules of beaten egg white. *a*, Globule with two rings. The peripheral ring had a reddish blue color, the inner ring was tinted blue. The cross bar stained light red. See arrow. *b*, Ruptured globule. The broken top ring, which is partially extruded, had a deep blue color and was seemingly superimposed on another ring, also broken, that was reddish blue in hue. The two other rings and the cross bar (at arrow) had the same tint as the latter ring.

#### THE NATURE OF CABOT RING BODIES.

The following experiments prove that the ring bodies are protein derivatives of the envelope and cytoplasm; and that the variability of affinity for acid and basic dyes is determined by the molecular thickness and momentary physico-chemical state of the protein mass.

*Experiments With Egg White.—Experiment 1.* The white of a raw egg was carefully separated from the yolk and beaten (or shaken in a test tube) until it was stiff. With the aid of a wire loop a small amount of the substance was transferred to a clean glass slide and spread to a thin film with a suitable spreader, cover slip, etc. The film was permitted to dry, then covered with Wright's blood stain for one minute, after which the stain was diluted with an equal amount of distilled water. The diluted stain was allowed to act for two to three minutes. The preparation was then rinsed in distilled water and permitted to air dry. Such preparations not only showed numerous single globules, but the globules varied also in size and thickness, and thus in some respects resembled normal and pathologic protein quantities of an erythrocyte, i.e., the globules are analogous to the erythrocyte envelope. The majority of the globules showed one or two, and less frequently three or more, reddish-blue rings, either concentrically arranged, or superimposed upon each other. Fig. 7 shows two representatives of the globules; the globule to the right, ruptured either during the drying process or during the staining procedure, shows a striking difference in thickness of the rings; a replica of Cabot rings in every respect.

*Experiment 2.* A thin film of unbeaten egg white was made on a microslide, and the substance was exposed to room temperature for at least one hour and then stained with Wright's stain in the usual way. In such a preparation there are areas in which many various-sized round to oval structures may be seen that show generally one concentric complete ring, and variable types of incomplete structures. A typical example is shown in Fig. 8, a replica in every respect of Fig. 1. When two thin films are superimposed upon each other, the formation of a figure eight is frequently encountered. Fig. 9 shows the manner in which such a configuration is produced. These protein structures are broader and flatter than those produced with beaten egg white.



Fig. 8.



Fig. 9.

Fig. 8.—Protein ring bodies of unbeaten egg white. *a*, Spherical body with deep blue flat ring. Note the uniformity of staining of the outer and inner area of the mass. A striking simulacrum of Fig. 1*a*.

Fig. 9.—Protein configurations of two thin films of unbeaten egg white. Incomplete figure eight. Note that two spherical bodies at different planes are involved in its formation.

Thus variability of dimensions, i.e., thickness of Cabot ring bodies, depends upon the availability of protein within the envelope or cytoplasm of a given erythrocyte.

The experiments showed that protein, regardless of its source, forms predominantly circular structures. The following hypothesis is offered for the presence of more than one ring body in an erythrocyte:

It is assumed that the human red blood cell envelope is not homogeneous, but consists of a series of concentric lipoprotein layers which under certain conditions may separate, in contrast to the cytoplasm which is a homogeneous mass, and thus merely retracts, behaving the same way as a thin layer of unbeaten egg white. Thus it is not at all difficult to account for the occurrence of more than one Cabot ring body at the same or at different planes within an erythrocyte, as well as for the thickness and configuration of the protein structures, as shown in Plate I.

#### DIFFUSE AND PUNCTATE BASOPHILIA

It is a well-known fact that Cabot ring bodies may appear in an erythrocyte showing either diffuse or punctate basophilia. Pappenheim<sup>39</sup> believed that polychromatophilia, punctate basophilia, and reticulation represent diffuse or aggre-

gate states of the spongioplasm. Many investigators, among them Key, Daum, and Cooke, have shown that Pappenheim's hypothesis was based on sound observations. Key<sup>40</sup> proved by appropriate methods that a basic substance retained by certain erythrocytes may be made to appear either as polychromatophilia, reticulation, or punctate basophilia, the latter representing an aggregation of the basophilic substance in the cell due to a pathologic process. Daum<sup>41</sup> showed that punctate basophilia may be created *in vitro* by permitting normal defibrinated blood to stand at room temperature for twenty-four to thirty-six hours. She observed that as autolysis progressed, diffused polychromatophilia changed to stippling, and as time went on, the punctate basophilia became more and more coarse, apparently in direct proportion to the autolysis. Daum came to the conclusion that the basophilic substance is most likely a colloid that is denatured and coagulated by heat, alcohol, or any other agent. Pappenheim thought the substance to be a lipid, as opposed to others who assumed the basic substance to be albuminous. Cooke,<sup>42</sup> using the blood smear method, employed a benzidine-alcohol-hydrogen peroxide mixture for the demonstration of punctate basophilia and reticulation in normal mature erythrocytes. He reached the following conclusion: "When obtained by the usual staining methods, this would point to increased permeability or a defect in the lipid envelope of the erythrocyte. There may be, and probably is, a close relationship between punctate basophilia, diffuse polychromatophilia, diffuse polychromasia, and reticulation and maturity." Cooke's conclusion supports the observation of Falconer,<sup>43</sup> of the occurrence of basophilic stippling in mature erythrocytes in the blood of normal healthy persons. On analysis of the evidence derived from the literature, it may be assumed that basophilia, polychromatophilia, reticulation, and punctate basophilia (stippling) are nothing more than the morphologic expression of denatured and coagulated colloid substances, i.e., lipoproteid colloids.

#### THE PRODUCTION OF DIFFUSE AND PUNCTATE BASOPHILIA WITH EGG YOLK

The experiments with egg white did not produce the phenomenon known as diffuse and punctate basophilia, and thus Pappenheim's assumption that lipoids may be responsible, was apparently supported. The following experiments were designed to obtain information concerning the role played by lipoids.

*Experiment I.* A thin layer of unbeaten egg yolk was made on a microslide, air dried, and stained with Wright's blood stain. The preparation revealed a homogeneous layer of a more or less reddish-purple hue.

*Experiment II.* A thin film of unbeaten egg white was made on a microslide and permitted to dry at room temperature. A thin layer of unbeaten egg yolk was superimposed and permitted to dry. The preparation was tinted with Wright's blood stain, air dried, and mounted in Clarite. Numerous round and oval bodies were seen that showed the well-known tinctorial characteristics of diffuse and punctate basophilia. The experiment showed that the presence of a protein layer enhances aggregation of lipoids. Fig. 10 shows several of these bodies. Thus the following opinion may be expressed: 1. The various tinctorial shades of diffuse basophilia are apparently the expression of a more or less

homogeneous state of protein and lipoids. 2. Punctate basophilia may be interpreted as the result of disassociation of these substances, or the independent reaction of the laminated lipid component.

Thus it is understandable why a ring body can appear in an erythrocyte exhibiting either diffuse or punctate basophilia. Fig. 11 shows a polychromatic red blood cell with a faintly stained ring body and punctate basophilia superimposed.

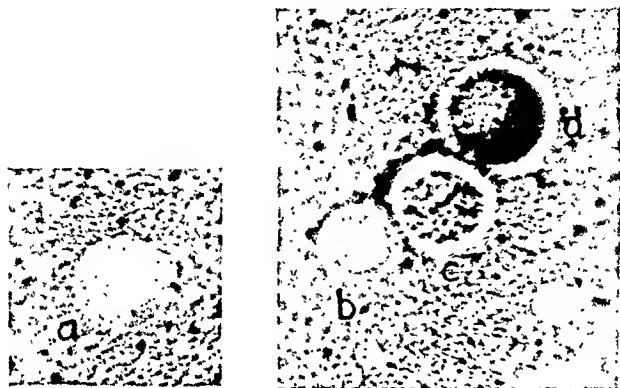


Fig. 10.

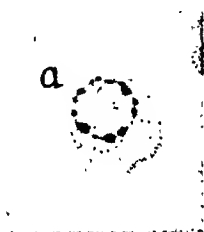


Fig. 11.

Fig. 10.—Egg white and egg yolk superimposed upon each other. *a*, Bluish round body resembling a basophilic erythrocyte. *b*, Round body (slightly out of focus) with fine granules which stained reddish blue. *c*, Round body with coarse blue granules. A striking replica of punctate basophilia. *d*, Round body with coarse deep blue and reddish blue granules. The white zone around the bodies is the result of retraction of the egg white.

Fig. 11.—Mr. R. P. Acute lead poisoning; peripheral blood. *a*, Orthochromatic erythrocyte with a light red protein ring and superimposed dark blue globules.

#### EXPERIMENTS WITH NORMAL HUMAN ERYTHROCYTES

As previously pointed out, the prerequisite for the formation of Cabot ring bodies is a physicochemical imbalance of the protein-lipoid components of the cell, permitting disintegrating agents to permeate and to precipitate the stroma protein. While these constituents are properly balanced, they form a definite structural unity. The process which supposedly modifies the envelope of the red blood cell is called "hemolysis," assumed to be a reaction between a lysin and the protein. It is obvious that the production of a simulacrum of the Cabot ring bodies in normal erythrocytes is possible only if the erythrocyte is exposed to a hemolysin that modifies the permeability of the envelope. If colloidal proteid is the substance that through denaturation forms a ring structure, then the problem at hand is to set up experiments that first assured hemolytic action, i.e., conditioning of the envelope in such a manner that denaturation of the protein is assured, followed by the penetration of a dye which would stain the protein.

The oldest and most effective means of altering envelope permeability is heat. This thermal factor, while a weak hemolysin, is responsible for many artifacts in cytology, but under controlled conditions is a valuable agent, especially when combined with strong alcohols and weak acids. The investigations carried out by Foà<sup>44</sup> on mammalian and human blood, and by Dehler<sup>45</sup> on domestic fowl, brought out the fact that the entrance of dissolved substances, such as dyes, into



the envelope and cytoplasm, is more rapid at high temperatures than at low temperatures. This knowledge has been utilized in devising an experiment for the creation and staining of replicas of "synthetic" Cabot ring bodies. While studying the variability of the envelope resistance, I consulted the papers by Weidenreich<sup>46</sup> and Sakai.<sup>47</sup> The latter, as already pointed out, produced ring bodies, but failed to suggest their similarity to Cabot rings. The striking replicas obtained by Sakai made his method the one of choice for preliminary work. The method, however, had to be modified for the use of human erythrocytes. Below is Sakai's method as I modified it:

#### I. Solutions

1. Ethyl alcohol, absolute.
2. Phenol (crystals C.P.) 2 per cent aqueous solution.
3. Phenol-gentian violet solution. (Add 5 Gm. of gentian violet to 100 c.c. of pure acetone-free methyl alcohol.)  
Take 40 c.c. of solution (2) and 10 c.c. of solution (3), mix well, and let stand at room temperature for 24 hours. Filter and transfer to a staining jar. The dye is good for only 24 to 36 hours.

#### II. Method

1. Make a thin blood smear and let air-dry for 5 hours.
2. Place into ethyl alcohol for 5 minutes.
3. Place into phenol solution 2 per cent for 30 seconds.
4. Place into phenol-gentian violet for 2 minutes.
5. Rinse in distilled water for 15 seconds.
6. If counterstain is desired, cover preparation with diluted Giemsa solution for 5 minutes.
7. Rinse in distilled water and let air-dry.
8. Mount with dammar or Clarite.

In a blood smear subjected to the foregoing treatment, the erythrocytes have a bluish tint, and their contour is sharply defined and not distorted. At the periphery a narrow, somewhat lighter area follows the shape of the cell. This phenomenon is apparently a sequela of the formation of a ring body; it can be seen in Fig. 3, cell *b*. A well-defined flat and dark-stained blue ring structure, at some distance from the periphery, is present in every mature erythrocyte, a striking replica of a Cabot ring body (Plate I, 1). The ring structure seems to be derived from a layer or layers at or near the surface, or adjacent to the envelope. Frequently two ring structures may be seen. Cell degeneration is thus seemingly expressed as complete or incomplete rings, or a ring is suggested by a ringlike arrangement of beads or rods. Cell immaturity can be recognized by the decreasing definition of the ring structure, which eventually is replaced by more or less fine granules which may fuse and form coarse clumps. The percentage of the latter types corresponds to that of the reticulocytes, Fig. 12, cells *c*, *e*, *f*. The amount of basophilic spongioplasm within the cell apparently determines the respective pattern. These standard patterns are thus the expression of the physicochemical status of an erythrocyte. In normal basophilic erythroblasts the spongioplasm is diffuse and deeply staining. Fig. 12 shows a characteristic pattern obtained with the method. Fig. 13 shows an erythroblast in mitosis, late in telephase stage. The lipoproteid colloids may well be in a transition stage, i.e., the respective quantities are as yet not properly assimilated. If this is the case, then the question of why Cabot ring bodies are so rarely seen

in erythroblasts is answered. However, bizarre patterns may be observed in severe anemic bloods, especially when cytolysis of erythroblasts is pronounced. When such blood is exposed to the phenol-gentian violet solution, the erythroblasts may occasionally show incomplete rings, but generally, fine or coarse granules, rods, spotted or diffuse spongioplasm, or combinations of the enumerated patterns may be seen. When in a routinely stained blood smear an erythroblast with a Cabot ring body is observed, the cell is most likely abnormal, as shown in Fig. 14.

The experiment revealed that replicas of Cabot ring bodies may be produced in normal mature erythrocytes but not in normal erythroblasts. The creation of the bodies depends upon the physicochemical state of the lipoprotein colloids at the various maturation stages. Further, an explanation is furnished for the rare occurrence of Cabot ring bodies in erythroblasts. There is no doubt in my mind of the identity of the simulacra created by the afore-mentioned method and the Cabot ring bodies. Both structures are the expression of denatured protein.

#### GLACIAL ACETIC ACID METHOD FOR THE CREATION OF DENATURED PROTEIN CONFIGURATIONS (CABOT RING BODIES) IN NORMAL MATURE ERYTHROCYTES

This method was based upon the well-known facts that weak acids and strong alcohols are hemolysins which in turn denature protein. The method has the advantage of reducing the room temperature exposure time. Heating the stain was found to facilitate rapid penetration.

##### I. Solutions

1. Wright's blood stain.
2. Glacial acetic acid 0.02 per cent.
3. Clarite or dammar.

##### II. Method

1. Permit the blood smear to air-dry at room temperature for one hour.
2. Place 20 drops of Wright's blood stain in a test tube, and heat over a flame to 50° C. Pour the dye quickly over the blood film, starting at the thick end.
3. Dilute the dye with 60 drops of glacial acetic acid 0.02 per cent. Mix well with an eye dropper by drawing in and expelling the dye.
4. Direct a flame under the glass slide and heat the dye solution to 50° C. This temperature is generally reached when the solution begins to steam.
5. Permit the hot dye to react for one minute. Rinse in distilled water for a few seconds and then let the preparation air-dry.
6. Mount with dammar or Clarite.

With the method described above patterns resulted identical to those created with the phenol-gentian procedure. When, however, the stain and acetic acid are heated together and permitted to act about one minute, many interesting patterns are obtained. Fig. 15 shows an example pattern that is produced with this modified procedure when blood samples of severe toxic and chronic anemias are used. The incomplete removal of the hemoglobin is responsible for the bizarre patterns. There is no erythroblast in the field.

#### DISCUSSION

While Cabot suggested that the ring bodies he described may represent nuclear remnants, it seemingly remained for Ferrata, Viglioli, and others to show



Fig. 12.—Mrs. B. W. Pernicious anemia in severe relapse. Peripheral blood treated with the author's acetic acid method. *a*, Normal mature erythrocyte with a characteristic protein ring. Note the striking resemblance to Fig. 1*a* and Fig. 8*a*. *b*, Mature erythrocyte with a ring body and a globule. *c*, Macro-erythrocyte with two ring bodies. The peripheral ring was light red, the other one was partially red and blue. Note the cross bars connecting the rings. A replica of the bars is seen in Fig. 7*a* and *b*. *d*, Macro-erythrocyte with incomplete light red rings and two blue globules. *e*, Megalocyte with incomplete ring, two globules, and a bar. The granular background is basophilic substance. *f*, Megalocyte (reticulocyte stage) more immature than cell *e*. The cytoplasm has retracted away from the periphery, leaving a light zone. *g*, Erythrocyte with the envelope ruptured. *h*, Thrombocytes.

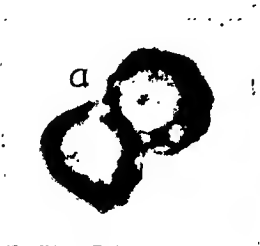


Fig. 13.

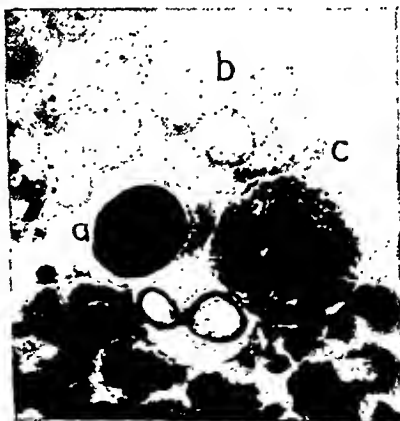


Fig. 14.

Fig. 13.—Mr. F. M. Normal bone marrow treated with the acetic acid method. *a*, Erythroblast, late telophase. Note the basophilic spongioplasm, sharply defined nuclear membrane, and blotches in the cytoplasm. The constituents of the envelope and cytoplasm arc seemingly not properly assimilated, and this may account for the absence of rings in erythroblasts. In the few exceptions cited, it is assumed that protoplasmic maturation was further advanced and that this led to the production of ring bodies.

Fig. 14.—Mr. A. K. Generalized Hodgkin's disease; peripheral blood. *a*, Pathologic polychromatic erythroblast with two twisted protein rings. The darker structure had a deep red color; the lighter one, lying at a lower plane, had a blue hue. *b*, Markedly lysed polychromatic erythrocyte with a red protein ring. *c*, Ruptured erythrocyte with the cytoplasm flowing around cell *b*. The ring had a reddish blue tint and appeared to belong to the envelope.

that the structures are identical with the nuclear membrane. This prevailing view, however, is based on misinterpretation of artifacts so commonly encountered in cytology. The literature dealing with the subject is extensive, and contains many diametrically opposed theories regarding the origin and nature of the Cabot ring bodies. None of the investigators has furnished convincing proof that the structures are either nuclear derivatives or artifacts, and thus laboratory creations. I have attempted to show that the Cabot ring bodies are actually the expression of denatured protein, i.e., colloid protein, and thus that the bodies have no immediate relation to the nuclear membrane. The ring bodies created at will in the laboratory, by slanting the glass slide while making the blood smear, and thereby delaying the drying process in a certain area, are in every respect identical with the genuine structures.

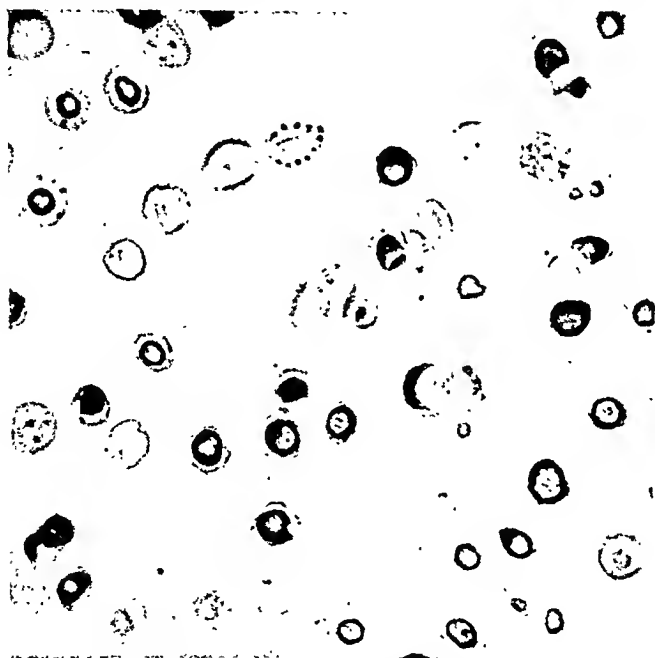


Fig. 15.—Mr. G. E. Chronic toxic anemia (carcinoma of the cecum), treated with the acetic acid method (modified). The variability of the erythrocyte envelope to lytic agents is well demonstrated. In some cells which have been partially hemolyzed the hemoglobin simulates nuclear dissolution (Ferrata). Other cells conform to Demel's theory. There is no erythroblast in the field.

The replicas were produced in the anemic blood of patients with pernicious anemia, acute lead poisoning, terminal lymphosarcoma, acute malaria, terminal lymphatic leucemia, congenital hemolytic icterus during hemolytic crisis, erythroblastosis fetalis, and terminal malignant Hodgkin's disease. According to the literature, these toxic and hemolytic anemias furnished, for the most part, the material for Cabot ring bodies in routine blood smears, and thus there can be little doubt that hemolysis plays an important role as a preconditioning factor for the production of the structures.

It has been shown that Cabot ring bodies can occur only in cells in which the permeability of the envelope has been altered by a specific lysis. The reason for the preponderance of flat circular bodies lies in the assumed geometry

of the erythrocyte and the tendency of protein to aggregate into a ring structure. The rarity of Cabot ring bodies in erythroblasts is assumed to be due to either a peculiar quantitative or a qualitative state of the lipoproteid colloids, or these constituents at this particular developmental stage have not yet assumed their mature relationships. An explanation has been offered for the presence of both a ring body and punctate basophilia in the same erythrocyte by showing that ring structures are aggregations of protein and punctate basophilia is identified with lipoids. Simulacrum of both phenomena were reproduced with egg white and egg yolk, and by special methods in normal mature erythrocytes.

The formation of a Cabot ring body is seemingly as follows: a specific hemolytic agent (bile acid) affects a certain erythrocyte whether basophilic, polychromatic, or orthochromatic; the lysis is injurious to the envelope, i.e., it produces changes either qualitative or quantitative, or both, in the lipoprotein constituents of the surface layer which not only influences the permeability but also may lead to microscopic tears in the envelope. This physicochemical change encourages disassociation of the lipoproteid layers, leading to a separation of the normally fused layers into individual ones, or to the separation of a mass of fused layers. This irregular splitting produces the variability of flatness and thickness of the ring bodies. This segregation of protein layers occurs during the drying process, leading either to the formation of the protein configurations known as Cabot ring bodies, or to such structures as globules or bars, as shown in Plate I, 5 and 10. The answer to Cabot's query "why," the reddish blue, red, or blue color of the ring bodies lies in the very nature of the structures.

#### CONCLUSION

The origin and nature of the Cabot ring bodies of erythrocytes have been discussed and demonstrated by special methods. Cabot ring bodies are neither nuclear remnants nor are they identical with the nuclear membrane; they are laboratory creations, the expression of cellular degeneration induced by hemolytic agents, i.e., they are the expression of aggregated and denatured colloid protein.

I wish to thank Dr. Hal Downey, who offered numerous helpful suggestions, and who was kind enough to edit the manuscript; Dr. R. A. Gortner, Head of the Department of Agricultural Biochemistry, University of Minnesota, for his aid in connection with the chemical aspect of the problem; F. N. Ruslander, medical photographer, Detroit, Mich., for the care exercised in preparing the photomicrographs. I am also grateful for the personal communications of the late Dr. R. H. Jaffé, whose genuine interest in the problem stimulated the investigation.

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# THE TREATMENT OF GONORRHEAL URETHRITIS IN THE MALE WITH SULFONAMIDE DERIVATIVES\*

## A STUDY OF 199 CASES

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IT IS AN established fact that the use of sulfanilamide in gonococcal infections is not indicated since the later sulfonamides have been introduced. Pelouze<sup>1</sup> states that this substance was effective in only 25 to 40 per cent of over 900 cases treated by the Cooperating Clinics of the United States Public Health Service. Excluding sulfanilamide from the therapeutic armamentarium, there are three sulfonamide derivatives whose value has been established in the treatment of gonorrheal urethritis in the male. These substances are sulfapyridine, sulfathiazole, and sulfadiazine.

The authors have previously reported their experiences with these three substances in the treatment of gonorrhea in the male.<sup>2-5</sup> Though the number of cases we treated is not large, we believe that we are able to make some comparison of the therapeutic efficiency and action of these three sulfonamide derivatives. The basis for this report is the study of 199 patients with gonorrhea, who presented themselves for treatment, at the Genito-urinary Clinic of the Philadelphia General Hospital during the years 1939 to 1941.

Of the 199 cases of gonorrheal urethritis studied in the male, 167 were followed to the final step of either apparent cure or failure, while 32 patients defaulted. The average age of the 199 patients was 25 years, but their ages ranged from 11 to 54 years. One hundred and seventy-two of the patients were black, 26 were white, and one was Chinese. One hundred and forty-three had no treatment of any kind before coming to our clinic, while 56 had some type of treatment. Twenty-six had purchased sulfanilamide from druggists. One hundred previously had a gonorrheal infection, while 99 never had gonorrhea.

The classification of gonorrhea in the male, which was used in this study, is that of Eisendrath and Rolnick. They classify gonorrheal infection as follows<sup>6</sup>:

- Acute urethritis (up to 3 months)
  - Anterior
  - Posterior
- Subacute urethritis (3 to 6 months)
  - Anterior
  - Posterior
- Chronic urethritis (6 months or more)
  - Anterior
  - Posterior

\*From the Genito-urinary Clinic, the Philadelphia General Hospital, Philadelphia.  
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Of the 199 patients who presented themselves to our clinic for treatment, there were 130 with acute anterior urethritis, 59 with acute posterior urethritis, 3 with subacute anterior urethritis, 6 with subacute posterior urethritis, and one with chronic posterior urethritis. Of the 156 patients who were eventually cured by these sulfonamide derivatives, 18 had epididymitis, 4 had gonorrheal arthritis, 4 had balanoposthitis, one had a perineurethral abscess, and one had inguinal adenitis before therapy was begun.

The diagnosis of gonorrheal urethritis was based on the history, clinical symptoms and signs, and positive bacteriologic studies. These bacteriologic studies consisted of a smear and culture of the urethral exudate in every case. All stains were done by the Gram technique. The cultural technique used to identify the gonococcus was as follows<sup>\*</sup>:

The urethral exudate or prostatic fluid is collected on a cotton swab. This swab is previously moistened with sterile physiologic saline. The swab is streaked on chocolate blood agar plates,<sup>†</sup> which are then incubated<sup>‡</sup> at a temperature of 37.5° C. for twenty-four to forty-eight hours. At the end of this period typical colonies are isolated and placed on chocolate blood agar slants. These slants are incubated at the same temperature for twenty-four to forty-eight hours. The culture on the slant is tested for purity by the Gram stain technique, and sugar tubes, containing dextrose, maltose, and levulose with a broth base, are incubated at the same temperature for twenty-four to forty-eight hours at 37.5° C. Gonococci ferment only dextrose with the production of acid and no gas; they do not ferment the other sugars. From the chocolate blood agar slants the organisms are also tested by the Alkali Solubility Test.<sup>§</sup>

The patients were seen twice a week during the early stages of treatment, and later at intervals of one or two weeks. At each visit the customary urologic examinations were made, including bacteriologic work at appropriate time intervals. Complete studies of the cellular elements of the blood were made in 55 cases before and after treatment with the drugs. Electrocardiographic tracings were taken before and after treatment in 51 cases. Blood levels of the sulfonamide were made on almost every patient. The technique of Bratton and Marshall was used in these analyses.<sup>¶</sup>

In the beginning of the study it was decided to keep the dosage as uniform as possible, yet to individualize the dose to fit the patient's disease. The drugs were continued for a few days after the patient was free of discharge or the urine had become clear in both glasses.

All cases received the drug in divided doses, usually two, three, or four times a day. With sulfapyridine the average dose to effect a cure was 23.5 Gm. given

<sup>\*</sup>This technical aspect of the study was done by Drs. H. A. Shelanski and L. Shelanski, of the Department of Zoology, the University of Pennsylvania.

<sup>†</sup>The composition of chocolate blood agar is as follows: Difco proteose No. 3 agar. Add 0.3 per cent whole defibrinated horse blood. Add blood when agar is 70° to 75° C. to get chocolate agar.

<sup>‡</sup>All cultures are placed in an atmosphere of 10 per cent carbon dioxide and an excess of moisture when they are incubated.

<sup>§</sup>The procedure of the alkali solubility test is as follows: 0.1 N sodium hydroxide is added to a suspension of bacteria. If the bacteria are gonococci, there will be complete lysis of the organisms. This does not occur if the organisms are meningococci or *N. catarrhalis*.



over a period of eight days. The range of dosage was 14 to 111 Gm. given over a period of from seven to thirty-seven days. With sulfathiazole the average dose to effect a cure was 28 Gm., given over a period of eight days. The range of dosage was 12 to 48 Gm., given over a period of four to twenty-eight days. With sulfadiazine the average dose to effect a cure was 17.5 Gm., given over a period of eight days. The range of dosage was from 4 to 58 Gm., given over a period of from four to twenty-one days (see Table I).

TABLE I  
CHEMOTHERAPY IN GONORRHEAL URETHRITIS IN THE MALE  
SUMMARY OF ESSENTIAL DATA

|   | SULFAPYRIDINE<br>57 CURED CASES                                     | SULFATHIAZOLE<br>48 CURED CASES                                  | SULFADIAZINE<br>51 CURED CASES                                    |
|---|---|--|---|
| Dosage to effect a cure   | Average: 23.5 Gm. in 8 days<br>Range: 14 to 111 Gm. in 7 to 37 days | Average: 28 Gm. in 8 days<br>Range: 12 to 48 Gm. in 4 to 28 days | Average: 17.5 Gm. in 8 days<br>Range: 4 to 58 Gm. in 4 to 21 days |
| Duration of discharge   | Average: 3 days<br>Range: 2 to 8 days                               | Average: 3 days<br>Range: 1 to 7 days                            | Average: 4 days<br>Range: 1 to 30 days                            |
| Time elapsing from beginning treatment until appearance of first negative prostatic culture                     | Average: 51 days<br>Range: 14 to 162 days                           | Average: 28 days<br>Range: 7 to 88 days                          | Average: 13 days<br>Range: 3 to 43 days                           |
| Time elapsing from cessation of symptoms until last positive culture of the prostatic fluid (the carrier state) | Average: 17 days<br>Range: 2 to 50 days                             | Average: 17 days<br>Range: 3 to 53 days                          | Average: 3 days<br>Range: 0 to 18 days                            |

The provocative tests were begun when clinical symptoms had ceased, and the urine had remained clear for three or four days. These tests were begun early because of the type of patients with which we were dealing. Most of the patients had a tendency to default when they began to feel that they were improving. The following consecutive tests were required of a patient before he could be discharged as cured: (1) Prostatic massage, smear and culture of fluid; (2) alcoholic indulgence; (3) passage of a sound into the urethra; (4) examination of a condom specimen; (5) repeated prostatic massages, both smear and culture of the prostatic fluid to be negative for gonococci on two or more occasions.

The results of treatment of gonorrheal urethritis in the male with the three sulfonamide derivatives are summarized in Table II. Of the 199 patients studied, 32 (16.2 per cent) defaulted and 167 (83.8 per cent) were followed to the completion of the cure, or failure. Of the 167 patients followed, 156 (93.5 per cent) were apparently cured, as evidenced by satisfying all criteria of cure, and 11 cases (6.5 per cent) were failures. Most of the patients treated had anterior urethritis, but all forms of acute and subacute urethritis were represented in this study.

Of 62 cases treated with sulfapyridine and followed, 92 per cent were apparently cured. Of 50 cases treated with sulfathiazole and followed, 96 per cent

were apparently cured, and of 55 cases treated with sulfadiazine and followed, 93 per cent were apparently cured. There is no significant statistical difference between these cure rates.

TABLE II

TREATMENT WITH SULFONAMIDE DERIVATIVES OF GONORRHEAL URETHRITIS IN THE MALE  
AN ANALYSIS OF 199 CASES

Philadelphia General Hospital, 1939-1941

|               | TOTAL<br>CASES | CASES<br>FOL-<br>LOWED | CURED CASES |      | FAILURES |     | DEFAULTERS |      |
|---------------|----------------|------------------------|-------------|------|----------|-----|------------|------|
|               |                |                        | NO.         | %    | NO.      | %   | NO.        | %    |
| Sulfapyridine | 87             | 62                     | 57          | 92   | 5        | 8   | 25         | 28.8 |
| Sulfathiazole | 55             | 50                     | 48          | 96   | 2        | 4   | 5          | 9    |
| Sulfadiazine  | 57             | 55                     | 51          | 93   | 4        | 7   | 2          | 3.5  |
| Total cases   | 199            | 167                    | 156         | 93.5 | 11       | 6.5 | 32         | 16.2 |

The results of treatment were uniformly good, no matter what the existing pathologic condition was at the time of beginning treatment. There were no complications in any cases during the period of treatment.

Analysis of the cured cases showed the average number of visits to the clinic before cure was complete to be eight for sulfapyridine, six for sulfathiazole, and eight for sulfadiazine.

In the entire series 11 cases were treatment failures. With sulfapyridine there were 5 failures. Failure in 2 cases was due to sensitivity to the drug that resulted in exfoliative dermatitis. The other 3 failures with sulfapyridine were patients who failed to cooperate during the period of treatment. They had coitus and alcoholic debauches during the early stage of treatment. With sulfathiazole there were 2 failures. These 2 patients failed to cooperate during the treatment period. With sulfadiazine 4 cases were treatment failures. It is interesting to note that 2 of these cases were syphilitic and were receiving mapharsen 0.06 Gm. per week at the same time that they were receiving sulfadiazine. The poor results in these two cases would bring up the question as to whether or not mapharsen has an inhibiting effect on the action of sulfadiazine. One of the failures had previously been a therapeutic failure with sulfanilamide and sulfathiazole. At the time that he began treatment with sulfadiazine he had a subacute anterior-posterior urethritis and prostatitis refractory to the two sulfonamides mentioned. The other failure did not cooperate.

The incidence of those who defaulted with sulfapyridine was 28.8 per cent, with sulfathiazole 9 per cent, and with sulfadiazine 3.5 per cent. The differences in default rate may be related to differences in the toxicity of the three drugs.

Toxic reactions were more commonly observed with sulfapyridine than with any of the other drugs. Seventy-five per cent of the patients treated with sulfapyridine complained of symptoms of toxicity, 11.5 per cent with sulfathiazole, and only 8.8 per cent with sulfadiazine. Three cases treated with sulfapyridine developed severe exfoliative dermatitis requiring hospitalization. These three cases were previously reported in detail.<sup>8</sup> Headache was the most common complaint with all three drugs. Complaints were severe among those receiving sulfapyridine, but minor among those taking sulfathiazole and sulfadiazine.

In the cured cases the time elapsing between the beginning of specific therapy and the cessation of discharge for sulfapyridine averaged three days, and ranged from two to eight days; for sulfathiazole, it averaged three days, and ranged from one to seven days; and for sulfadiazine it averaged four days and ranged from one to three days (see Table I).

The time that elapsed between the beginning of specific therapy and the first culture of the prostatic fluid which was negative for the gonococcus showed interesting differences among the three drugs. This period of time for sulfapyridine averaged fifty-one days, and ranged from fourteen to one hundred and sixty-two days; for sulfathiazole it averaged 28 days, and ranged from seven to eighty-eight days; and for sulfadiazine it averaged thirteen days, and ranged from three to forty-three days (see Table I).

The time elapsing from the disappearance of the symptoms of gonorrheal urethritis to the last culture of the prostatic fluid positive for the gonococcus is the period of time known as the so-called "carrier state." This period for sulfapyridine averaged seventeen days, and ranged from two to fifty days; for sulfathiazole it averaged seventeen days, and ranged from three to fifty-three days; and for sulfadiazine it averaged three days, and ranged from zero to eighteen days (see Table I).

Blood levels of the drugs, appearing with the dosages stated previously, were as follows: sulfapyridine, total, 1.6 to 9 mg. per 100 c.e.; sulfathiazole, free, 0.4 to 8.7 mg. per 100 c.e.; and total, 1 to 11 mg. per 100 c.e.; sulfadiazine, free, 1.5 to 10.4 mg. per 100 c.e.; and total, 2.1 to 11.5 mg. per 100 c.c. We did not find the blood levels of any prognostic value in regard to predicting toxic reactions or in determining the rapidity of cure.

The effect of the various sulfonamide derivatives studied on erythrocyte count, hemoglobin, leucocyte count, and differential count, was observed in 55 patients. In general, there were no significant changes.

Electrocardiographic studies were made on 51 patients before and after treatment with the drugs. There were no significant changes.

#### SUMMARY

A total of 199 patients were studied. Of this number 167 were followed to the completion of the study. Ninety-three and one-half per cent of these cases were cured. The rate of cure was 92 per cent for sulfapyridine, 96 per cent for sulfathiazole, and 93 per cent for sulfadiazine.\* There is no significant statistical difference in these cure rates.

To effect a cure, the average dose required was for sulfapyridine 23.5 Gm. in eight days, for sulfathiazole 28 Gm. in eight days, and for sulfadiazine 17.5 Gm. in eight days.

\*Five months' follow-up on the cases apparently cured by sulfadiazine revealed that of the 51 cases originally discharged as apparently cured, 8 had returned with reinfection. Each of these 8 cases had been observed for from twenty-eight to seventy-six days and had three to eight smears and cultures of the prostatic fluid negative for the gonococcus before being discharged as apparently cured. It is difficult to say definitely whether the reappearance of infection was due to recurrence of the old infection or to reinfection from a new contact reinfection as treatment failures have a history of exposure. If we are to count the 8 cases of reinfection as treatment failures our percentage of apparent cures with sulfadiazine would be 78 per cent instead of 93 per cent originally reported.

Toxic reactions from the drugs occurred in 75 per cent of the cases treated with sulfapyridine, 11.5 per cent with sulfathiazole, and 8.8 per cent with sulfadiazine.

The average time for the discharge to cease in patients treated was three days with sulfapyridine, three days with sulfathiazole, and four days with sulfadiazine.

The average time for a bacteriologic cure, based on the appearance of the first negative culture of the prostatic fluid, was fifty-one days for sulfapyridine, twenty-eight days for sulfathiazole, and thirteen days for sulfadiazine.

The "carrier state" so-called (the period from the disappearance of symptoms to the last culture of the prostatic fluid known to contain living gonococci) averaged seventeen days for sulfapyridine, seventeen days for sulfathiazole, and three days for sulfadiazine.

The criteria of cure in this study were strict and included as the final provocative test two or more negative smears and cultures of the prostatic fluid.

#### CONCLUSIONS

From our studies it would seem that sulfadiazine is a valuable drug in the treatment of gonorrheal urethritis in the male because it has a high cure rate and a low incidence of toxicity; it causes the prostatic fluid to become free of living gonococci sooner than any of the other sulfonamides we used.

Sulfathiazole is very effective therapeutically and causes few toxic reactions.

Sulfapyridine would better not be used in the treatment of gonorrhea in the ambulatory patient. It is very effective therapeutically, but it gives rise to many and severe toxic reactions.

We wish to acknowledge the cooperation of Dr. Jefferson Clark, chief of the laboratories; Dr. Walter Crocker, chief of the division of clinical pathology; Dr. John Reinhold, chief of the division of biochemistry; and Dr. Thomas McMillan, chief of the division of cardiology of the Philadelphia General Hospital.

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Sulfathiazole was furnished by the Maltbie Chemical Co., Newark, N. J.

Sulfadiazine was furnished through the courtesy of the Pneumonia Control Committee of the Philadelphia General Hospital, and the Nepera Chemical Co., Yonkers, N. Y.

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# PYELITIS WITH SEPTICEMIA CAUSED BY *S. PARADYSENTERIAE*\*

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COMPARATIVELY few cases of septicemia or of urinary tract infections due to paradysentery bacilli have been reported. Our case seems to be of special interest because both pyelitis and septicemia caused by *Shigella paradysenteriae* complicated the post-partum course of the patient. The same organism was isolated from two stool specimens, although no diarrheal symptoms were observed.

Approximately 30 cases have been recorded in which a septicemia has been attributed to a dysentery bacillus. In 1926 Ashworth and others reviewed the literature on the occurrence of septicemia in cases of bacillary dysentery. He listed 24 cases, including the one which he reported, but among these were several which were not well proved. Rothman<sup>2</sup> in 1936 added one case to this list, and in 1937 one of us<sup>3</sup> reported two fatal cases of paradysentery in infants with positive blood cultures. Dodd and Swanson<sup>4</sup> in 1938 reported three cases of paradysentery from one of which a positive blood culture was obtained before death, and from the other two cases post mortem.

Fourteen cases of infection of the urinary tract by paradysentery bacilli had been recorded when the literature was reviewed by Neter<sup>5</sup> in 1937. In two of three cases which he added to this group, a paradysentery bacillus was isolated from the stool as well as the urine; in the other case apparently only from the urine. No blood cultures were made. In August, 1940, we had in our hospitals two cases, and in December, 1940, there were also two cases of urinary tract infection due to paradysentery bacilli. The blood cultures of the first two, and one of the second pair, all of which were adults, were negative, while none was taken from the fourth patient, who was a child.

Two cases reviewed by Neter,<sup>5</sup> that reported by Calalb and Ionescu and that reported by Cheatham, were cases of pyelonephritis complicating pregnancy.

## CASE REPORT

D. R., aged 41 years, para 0, gravida iii, was admitted to the William H. Coleman Hospital on July 5, 1940, when her pregnancy was approximately at term. She complained of epistaxis of three days' duration. Her physician had detected hypertension at the time of her first visit at the sixth month. Five and again two years previously, she had had spontaneous abortions at six months. Her last pregnancy was known to be complicated by a systolic blood pressure of 220. She gave a history of rheumatic fever as a child. At the age of 3 she had had a severe diarrhea, but no definite diagnosis was made. She had been employed as a nurses' aide in a mental institution from the age of 30 to 38. During this time she had suffered from a diarrhea of two days' duration. Many patients she had cared for had had diarrhea of an undetermined origin.

Physical examination upon admission revealed a markedly obese white female. The abdomen was enlarged to the size of a full-term pregnancy. Fetal heart sounds were heard.

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The retinae exhibited tortuosity of the retinal vessels, several small exudates, crossing phenomenon, and a vein-artery ratio of 2 to 1. A rough, low-pitched systolic murmur was heard loudest at the upper end of the sternum and the aortic region. The blood pressure was 220/110. There was slight edema of the lower extremities.

On admission the urine contained 0.521 per cent albumen, one granular cast per high-power field, and ten to twelve pus cells; blood counts were essentially normal. Eight hours following admission, the membranes ruptured spontaneously, and uterine contractions promptly ensued. Approximately ten minutes later no fetal heart sounds were heard. No abnormalities were found on vaginal examination. Labor was terminated at the end of twelve hours by the delivery of a full-term stillborn male infant. There was evidence of compression of the umbilical cord due to the peculiar manner in which the cord was wrapped about the infant's abdomen and both thighs. Ten hours' post partum the patient was seized by a severe pain in the right chest which markedly impaired respiratory movements. Examination revealed a friction rub in the area of pain, and a blood pressure reading of 165/100. Symptomatic treatment was instituted consisting of morphine and nasal oxygen. Four hours later a similar episode occurred, involving the lower portion of the left chest. Her pulse was of poor quality and her temperature was 100° F. rectally. A bedside flat plate of the chest was obtained, which showed only a haziness in the lower lung fields. The following day her condition appeared to be slightly improved; orally, her temperature was 102° F. On the third post-partum day her temperature rose to 104° F., and she had a chill. Her complaints then were referable to the pain in the chest, and no new physical findings were obtained. Another flat plate of the chest showed a clearing of the previously reported congested areas. From the fourth to the ninth days post partum, inclusive, she maintained a low-grade temperature elevation. On the tenth post-partum day the temperature which rose to 104.8° F. was accompanied by a chill and increased pain along the right costal border. On the eleventh post-partum day the temperature rose to 103.4° F. There were many clumps of pus cells and 1+ albumen in catheterized specimens of urine obtained these two days. A tentative diagnosis of pyelitis was made. A blood culture was obtained which yielded a gram-negative bacillus, which had typical cultural characteristics of the paratyphoid bacillus and was agglutinated by Hiss-Strong anti-serum to a dilution of 1:160.

The patient was given sulfanilamide therapy by mouth, following which there was general improvement in her condition. Examination of the fundi on the twelfth post-partum day revealed the presence of a recent flame-shaped hemorrhage in the right fundus. From the fourteenth to the twenty-eighth post-partum days the low-grade temperature elevation persisted, following which her temperature returned to normal and remained so thereafter for a period of twenty-two days. Her blood pressure stabilized at 140 systolic and 100 diastolic. Urea clearance was 51 per cent. X-ray studies made of the chest, urinary tract, gastrointestinal tract, and biliary tract failed to add any information to this case. A diagnosis of toxemia of pregnancy upon a vasculorectal basis, pulmonary embolism, septicemia due to a paratyphoid bacillus, and post-partum pyelitis was made.

A gram-negative bacillus identical with that obtained from the blood was obtained in pure culture from the urine on July 19, 1940, and was isolated from the stool on July 23, 1940. A second stool specimen on July 26, 1940, yielded the same organism. Blood cultures made on July 19, August 3, August 4, and August 6, 1940, were all negative.

Blood drawn from the patient on July 23, 1940, agglutinated *S. paratyphoid* Strong at 1:160 dilution and *S. paratyphoid* Hiss at 1:40 dilution. It agglutinated the organisms obtained from the blood, the urine, and the stool cultures at a dilution of 1:5,120. On August 20, 1940, the patient's blood serum agglutinated both Hiss and Strong dysentery bacilli at 1:160, and the organism isolated from the patient at 1:2,560 dilution.

Gram-negative bacilli, which seemed to be rough variants of the paratyphoid bacillus, were isolated from the urine and stool on August 20, 1940. These bacilli gave typical fermentation reactions in the test substances used, but in spite of repeated efforts, smooth strains, which would be suitable for agglutination tests, were not obtained from the cultures.

It seems possible that this patient was a carrier of the paradysentery bacillus and, since her resistance was reduced, an acute infection with the organism developed. The probable persistence of the organisms in the rough form after the acute symptoms had disappeared is of interest in this connection. Neter<sup>5</sup> concluded from his study of urinary tract infections caused by paradysentery bacilli, that these infections may occur during or following an attack of intestinal dysentery or in patients without clinical evidence or history of dysentery. It seems possible that the organism may persist in the genitourinary or intestinal tract after mild attacks of dysentery that the patient does not remember.

#### CONCLUSION

A case of septicemia and urinary tract infection due to *Shigella paradysenteriae* complicating the post-partum course of a patient has been reported. So far as we have been able to determine this is the first case to be reported of a paradysentery bacillus infection of the urinary tract with a positive blood culture.

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## SYNDROME SIMULATING DIABETES INSIPIDUS IN DOGS INDUCED BY DESOXYCORTICOSTERONE ACETATE\*

### CLINICAL OBSERVATION OF SYNDROME WITH ADDITION OF TETANY

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RECENTLY Ragan and associates<sup>1</sup> reported that with daily 25 mg. doses of desoxycorticosterone acetate injected into dogs for a period of six weeks, they were able to induce a syndrome of polydipsia and polyuria. Pitressin did not influence the syndrome, thus differing from true diabetes insipidus. We obtained similar results in dogs, using smaller doses in varying concentrations (5 and 10 mg.) over a period of four months. Some of the material used was dissolved in propylene glycol.†

Two of 5 dogs used developed the syndrome of polydipsia and polyuria, and one of these died.

Muscular weakness and lack of coordination were prominent features in all 5 dogs. Ragan and associates observed this in their dogs, and stated that it had previously been observed by Kuhlmann and others.

\*From the Department of Internal Medicine, Harper Hospital, Detroit.

†The material was kindly furnished by Dr. Max Gilbert, of the Schering Corporation. Received for publication, May 31, 1941.

The specific gravity of the urine in our polyuric dogs showed a gradual decrease toward 1.000. The intake of fluids was not accurately measured but all the experimental animals showed a marked increase in fluid intake.

Two dogs had outstanding symptoms of polydipsia and polyuria, being constantly thirsty and drinking large quantities of water. These two dogs died, while the others showed disappearance of the syndrome when the dosage was reduced to 5 mg. per day.

Soon after the syndrome was noted in the dogs, a male patient suffering from myasthenia gravis and in a relapse had a total pellet implantation of 1,300 mg. of desoxyeortieosterone acetate. One pellet of 150 mg. was purposely crushed. Within two weeks he developed a very marked polydipsia and polyuria. There was an increase of blood pressure from systolic 110 and diastolic 68 to systolic 138 and diastolic 92.

Coincident with the polydipsia and polyuria, there developed symptoms of tetany—a positive Chvostek sign and Trousseau's phenomenon was easily brought out during the reading of the blood pressure. He had spontaneous tetany of his hands several times during the day. Paresthesia of the extremities was also present. The serum calcium was 8 mg., and phosphorus was 3.93 mg. per 100 c.c. of blood. The electrocardiogram showed a prolonged RT-interval of 0.32 like that seen in hypoparathyroidism.

It is of interest that soon after the implantation the patient had a return of muscular strength, but when the polydipsia, polyuria, and tetany set in, symptoms of muscular weakness became marked. This was in distinct contrast to the striking improvement following the first implantation of the pellets. This muscular weakness differed from the original myasthenia gravis symptoms in that there was no recovery with rest; nor was there any speech difficulty. Further details of this will be given elsewhere. We know from our experimental data, as well as that of Ragan and associates, that this muscular weakness was in all likelihood due to overdosage of the drug.

The symptoms mentioned continued for two months, and for the last three months the patient states that he has never felt as well since the onset of his illness in October, 1939. A mild degree of polydipsia and polyuria are still present, but the tetany and paresthesia have disappeared. To our knowledge, this is the first instance in which the syndrome of polydipsia, polyuria, and tetany with paresthesia has been produced in a human being by overdosage with desoxyeortieosterone acetate.

The patient has repeatedly emphasized that the injections of the drug, while seemingly holding the disease stationary, do not compare with the relief obtained by implantation of the pellets. This is also the observation of Thorn.<sup>2</sup>

Our experiments confirm the findings of Ragan and his associates. They made careful electrolytic studies of their dogs in which they induced polydipsia and polyuria by giving daily injections of 25 mg. of desoxyeortieosterone acetate. While the symptoms were similar to diabetes insipidus, they noted that pituitrin was relatively ineffective, and fluid restriction did not cause dehydration in the animals. The converse is true in diabetes insipidus. They are of the opinion, therefore, that the condition which they described is primarily a thirst, and only secondarily a polyuria. Why this should be is not known, although speculative possibilities naturally present themselves.



Sections of various organs, such as the thyroid, adrenal, testes, ovaries, kidney, spleen, and muscle were all normal, except in one dog which showed deglycogenation of the liver. Separate sections of the hypothalamic area were made of the two dogs showing the polydipsic and polyuric syndrome. Unfortunately, the pituitary was lost during preparation for sectioning. The remainder of the pathologic report follows:

"In sections of one of the blocks there is a region in which the leptomeninges are infiltrated with plasma cells and in this region an adventitial infiltration of the intracerebral vessels is also seen. The infiltrating cells consist almost exclusively of plasma cells forming large coats around the lumen of the vessels. There seems to be an increase of blood vessels. However, this picture could be due to a more pronounced marking of pre-existing vessels because of adventitial infiltration. There is no visible adventitial endothelial proliferation. The region in which this definite pathologic finding was seen belongs to some part of the hypothalamus. It could not be determined which hypothalamic nucleus particularly is involved. The pituitary was not included in the blocks."

Summary: "The finding represents a localized encephalitic reaction showing a plasmacellular infiltration of the leptomeninges and the adjacent intracerebral blood vessels. Whether or not this encephalitic reaction is a part of a more generalized process cannot be determined because of lack of material."\*

These findings suggest certain correlations with the clinical picture of hypertension, polydipsia, and polyuria. This is based on the known physiology of the hypothalamus in its influence on blood pressure and water balance. Further studies along these lines are suggested by these data.

One of the dogs was pregnant and received the desoxycorticosterone acetate injections daily for six weeks and bore a litter of seven, only two of which were viable. One of the latter died within a week. One may conclude from this that the drug was probably responsible for the fetal deaths. We are not prepared to say as to the mechanism by which this was produced.

Selye's<sup>2</sup> work is of interest in this connection. He concluded that his experimental evidence indicated that both in the mouse and in the rat desoxycorticosterone acetate causes marked involution of the adrenal cortex. He stated that the atrophy of an endocrine gland caused by the administration of an excess of the hormone or hormones which it produces is regarded as the exact antithesis of the compensatory hypertrophy elicited by the hormone deficiency occasioned by partial extirpation of such a gland. For this mechanism of readjustment, Selye suggests the term "compensatory atrophy."

He is of the opinion that this is of clinical importance and says that, for example, the use of desoxycorticosterone acetate (a substance which is practically devoid of androgenic activity) in cases of adrenogenital syndrome in which there is an excess production of androgen in the adrenal may prove useful in inhibiting the faulty endocrine secretion of the cortex.

This would not be without its dangers, however, since in this syndrome hypertension is already present, and the known hypertensive effects of the desoxycorticosterone acetate would add fuel to the fire. Certainly the danger of

\*We wish to thank Dr. Gabriel Steiner from the Department of Pathology, Wayne University, for the pathologic report.

vaseular cerebral, and cardiac accidents, is to be considered, and this might occur before "compensatory atrophy" had set in.

The drug, no doubt, is a very useful one but must be used with caution, since it may give rise to dangerous, toxic symptoms.

#### SUMMARY

A syndrome of polydipsia and polyuria was induced in two dogs by comparatively large doses of desoxycorticosterone acetate. Profound muscular weakness was noted in the dogs so that they were unable to co-ordinate their muscles.

A pregnant dog which had received the drug for a period of six weeks gave birth to a litter of 7, 5 of which died, one lived for a week, and the remaining one survived.

Sections of the hypothalamus showed a localized encephalitic reaction with a plasmacellular infiltration of the leptomeninges and adjacent intracerebral blood vessels. Whether or not this encephalitic reaction is a part of a more generalized process cannot be determined because of lack of material. The suggestion was made that the clinical picture of polydipsia, polyuria, and hypertension might be understood by changes occurring in the hypothalamus.

A patient with myasthenia gravis, who had an overdosage of the drug by implantation of the pellets of this material and the crushing of one pellet, showed the syndrome of polydipsia and polyuria. Muscular weakness, such as was noted in the experimental animals, was also present. In addition, he had signs and symptoms of tetany, which we believe is a new symptom of overdosage.

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## THE EFFECT OF PITRESSIN ON RENAL CIRCULATION AND URINE SECRETION\*

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CONSIDERABLE work on human subjects and on various species of animals has been done in an effort to determine the action of pitressin on the circulation of blood in the kidney as well as on urine formation, and to explain the mechanism of antidiuresis. Diverse methods have been employed, and pituitary extracts containing various amounts of impurities have been administered. Consequently, considerable data have accumulated which manifest equivocal interpretations and contradictory conclusions. This paper is a presentation of an attempt to demonstrate the alterations in the glomerular tuft of capillaries and other vessels in the frog kidney, as seen under the microscope, and the changes in renal blood flow in the dog kidney under the influence of pitressin when administered through the various possible routes under similar conditions. The only preparation used for the work reported in this paper was pitressin in ampoules (1 c.c. = 20 units).

### A. EFFECT OF PITRESSIN ON THE GLOMERULAR CIRCULATION IN THE FROG KIDNEY

Adolph<sup>1, 2</sup> employed pituitrin (solution of posterior pituitary) as one of the methods of decreasing urine formation in the frog kidney. He observed that all the methods that diminished the production of urine decreased the circulation of the blood either locally or generally. He, therefore, suggested that these effects on the circulation account for the decrease in urine formation. By subcutaneous injection of pituitary extract into the frog (*Leptodactylus ocellatus*), Biasotti<sup>3</sup> found that in twenty-four hours the normal frogs had an average increase of 15.6 per cent in weight, and those with ligated ureters had an average increase of 26 per cent. He stated that Brunn<sup>4</sup> did not consider the effect to be produced by the kidney, because even in nephrectomized frogs the administration of pituitary extract causes a greater increase in weight than in the controls. Rey<sup>5</sup> injected posterior pituitary extract (Pituitobine Byla A or B) into the abdominal lymph sac of frogs (*Rana temporaria*), and observed a great diminution of urine formation for two or three hours after injection. He suggested that the posterior lobe extract modifies the three factors that take part in the regulation of water exchange in the body: namely, (1) the excretion by the kidney, (2) the retention by the tissues, and (3) the absorption by the skin.

On the other hand, the work of Collin and Drouet<sup>6</sup> casts doubt on the water retention action of the posterior pituitary extract. They injected the extract

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(Choay No. 2 and No. 4) into frogs and compared their weight changes, for forty-eight hours after injection, with those of controls under the same environmental conditions. Their findings indicated weight changes in those injected with extracts of the posterior pituitary lobe similar to the changes in the controls. Burgess, Harvey, and Marshall<sup>7</sup> accumulated evidence which enabled them to conclude that pitressin and pituitrin exert no antidiuretic action in the frog, but that in mammals the antidiuretic action of these substances is "due to a stimulation of water reabsorption by the thin segment of the loop of Henle." From his work on water interchange in frogs and their weight increases under the influence of pitressin, Steggerda<sup>8</sup> suggested the probability that pitressin causes an increase in skin permeability, possibly by acting directly on the circulatory system or by some change in the melanophores. He expressed the opinion that the antidiuretic effects of pitressin do not play a major role in the weight increases of the frogs. Richards and Schmidt<sup>9</sup> observed that small amounts of pituitrin administered intravenously increased the number of active glomeruli, and also the number of active capillaries in the tuft of a single glomerulus in the frog kidney; but both were decreased under the influence of large doses.

**Method.**—For our observations on the circulation in the frog kidney we modified the method of illumination described by Knisely.<sup>10, 11</sup> The experiments were made on anesthetized male frogs (*Rana pipiens*) whose weights ranged from 25 to 45 Gm. Anesthesia was produced by injecting into the dorsal lymph sac 0.1 c.c. of a 25 per cent solution of urethane per 10 Gm. of body weight. The abdomen of the frog was opened by a paramedian incision, carefully avoiding the big vessels, and the edges of the incision were gently cauterized to prevent loss of blood. The animal was laid on a block of cork with frog-Ringer's solution gently flowing over the exposed viscera to prevent drying. Water was kept trickling around the intact body of the frog to dilute the Ringer's solution in contact with the skin and to prevent dehydration. The kidney was protected from the heating effect of the light, transmitted through the quartz tubing, by a constant stream of Ringer's solution flowing through the bore in the delivery tip introduced retroperitoneally, dorsal to the kidney. Such a preparation could be kept functioning normally, without any signs of hemorrhage or other disturbances, for at least nine hours. Healthy frogs were used, and the normal kidney circulation in several microscope fields was carefully studied before any observations on the effects of pitressin were made. Amounts of pitressin ranging from 0.01 to 0.1 c.c. were injected either into the lymph sacs or intravenously into the anterior abdominal vein, or were applied directly through a very fine needle to the microscope field under observation. A stop watch was used to record the time when the drug was administered, when any effect was observed, and when the affected parts returned to their original condition previous to the administration of the drug.

**Results.**—When pitressin was administered by direct application of 1 or 2 drops over the microscope field under observation, it was found that in three to ten seconds undiluted pitressin produced cessation of activity in all glomeruli in the field. In some glomeruli the afferent arterioles contracted to disappearance during the maximal effect, and whatever blood cells were in the glomerular tuft of capillaries remained motionless until renewed activity gradually took

place. Such renewed activity would occur in a period ranging from two to ten minutes. However, not all the glomeruli ceased functioning at once, nor did they become active again at the same time. The afferent arterioles of some glomeruli contracted enough to prevent passage of blood cells while plasma still passed and gradually forced the corpuscles out of the glomeruli, thus leaving the glomerular tuft of capillaries free of blood cells until renewed activity took place. The blood flow in the other vessels of the kidney area under observation became so slow after application of pitressin that for a period of several minutes no axial or peripheral streams could be demonstrated, and the cells, white and red, could be easily identified in the slow stream throughout the lumina of the vessels. Dilutions of pitressin up to 1:100, when directly applied in 2 drops over the area under observation, gave the same changes but for much shorter duration. Higher dilutions gave no detectable change.

Injection of undiluted pitressin in amounts ranging from 0.05 to 0.1 c.c. into the lymph sac produced within two minutes a noticeable slowing of blood flow throughout the kidney areas under observation. Changes similar to those described under direct application of the drug were noticed, except that the glomeruli, instead of showing complete cessation of blood flow, showed very sluggish flow through their capillary tufts. This lasted for at least twenty minutes, and often for more than half an hour. Dilutions up to 1:200, when given in amounts of 0.1 c.c. into the lymph sac, produced a slowing of the blood stream in the various vessels of the kidney area, which was much less marked and shorter in duration than that produced by the undiluted dose.

The injection of 0.05 c.c. to 0.1 c.c. of undiluted pitressin into the abdominal vein of the frog produced, within twenty seconds, complete cessation of activity of all the glomeruli under observation. Some glomeruli became completely blanched and had no blood cells in their tuft of capillaries whatsoever, while other glomeruli had their capillaries packed with motionless red and white blood cells. This cessation of activity lasted for periods varying from two to eight minutes, after which recovery began to take place in the glomeruli at different times. The other vessels in the area showed marked slowing of the blood stream to such an extent that the flow became jerky, and in some vessels it wavered back and forth without much forward progress. The slowing of the blood stream in the kidney tissue under observation lasted from twenty to forty-five minutes. Dilutions up to 1:500 gave such effects as already described, but the duration generally varied inversely with the dilution. Dilutions higher than 1:500 produced no detectable changes. No matter what route was used for administration, undiluted pitressin produced various degrees of anemia of the area under observation as a result of vascular constriction.

#### B. EFFECT OF PITRESSIN ON RENAL BLOOD FLOW AND URINE SECRETION IN THE DOG

In a clinical investigation on human subjects, Stead, Kunkel, and Weiss<sup>12</sup> found that the intramuscular administration of pitressin produced a greatly reduced blood flow in the hands as well as abdominal cramps and ashen pallor. In their study on the trained dog, Geiling and co-workers<sup>13</sup> reported a marked and prolonged decrease of blood flow in the femoral and carotid vessels (arteries

and veins) following the intravenous administration of pitressin. However, in a few observations on the renal artery they observed a transient decrease followed by a prolonged and marked increase. Grindlay and two of us (Herriek and Mann<sup>14</sup>) reported a marked decrease in the blood flow of the spleen following the intravenous injection of 0.05 c.c. of pitressin into dogs. Essex and his co-workers<sup>15</sup> reported that the intravenous administration of pitressin into trained dogs produced a decrease from 64 to 84 per cent in the coronary blood flow and reduced the heart rate.

Handovsky and Samaan,<sup>16</sup> using the Rein thermostromuhr on both anesthetized and unanesthetized dogs, observed an initial reduction followed by a prolonged increase in renal blood flow under the influence of large doses of posterior pituitary extract administered intravenously. They used various commercial preparations of posterior pituitary extracts. Janssen and Rein<sup>17</sup> determined the renal blood flow of decerebrate dogs under the influence of fresh posterior pituitary extract. They concluded that the process of antidiuresis was not accompanied by any alterations in the renal blood flow. Walker and co-workers<sup>18</sup> injected pituitrin subcutaneously into unanesthetized rabbits and dogs, and determined the renal blood flow during the development of the antidiuresis. They observed very slight decrease in renal blood flow in some experiments, and an apparent marked increase in three of ten experiments. They stated that decrease in renal blood flow does not play any part in the antidiuresis in either dog or rabbit. Unpublished observations by A. E. Livingston and one of them, reported in a footnote, indicate a decrease in the size of the dog's kidney observed roentgenographically during pituitrin antidiuresis. They attributed this decrease to changes in tubule volume rather than to a decrease in the size of the vascular bed. Their observations on creatinine clearances indicated no consistent reduction under the effect of pituitrin, and they concluded that changes in the glomerular filtrate are not the mechanism of the antidiuresis.

In their work on dogs, Underhill and Pack<sup>19</sup> found that intravenous administration of pituitrin caused marked dilution of the blood, and that exclusion of the kidneys by mass ligature at the hilus previous to the injection of pituitrin did not modify the hydremia produced by the pituitrin. They considered the hydremia and the antidiuretic action to be due to some unknown action on capillary permeability. From his work on the isolated, perfused kidney of the dog, Verney<sup>20</sup> concluded that the pituitrin-like secretion picked up by the blood passing through the head inhibited the polyuria of the isolated kidney and decreased the renal blood flow. Richards and Plant<sup>21</sup> determined renal blood flow by the method of Barcroft and Brodie<sup>22</sup> in dogs, rabbits, and cats under urethane anesthesia after intravenous administration of small doses of pituitrin. They obtained a decrease in renal blood flow in dogs and rabbits but not in cats.

*Method.*—The observations on the renal blood flow were made by the thermostromuhr method of Rein, which was modified by Essex and two of us (Baltes and Herriek<sup>23, 24</sup>). Direct current thermostromuhr units were carefully calibrated before and after application. The calibrations were made and checked in such a way that the deflections of a sensitive moving coil galvanometer could be translated into cubic centimeters of blood flow per minute.

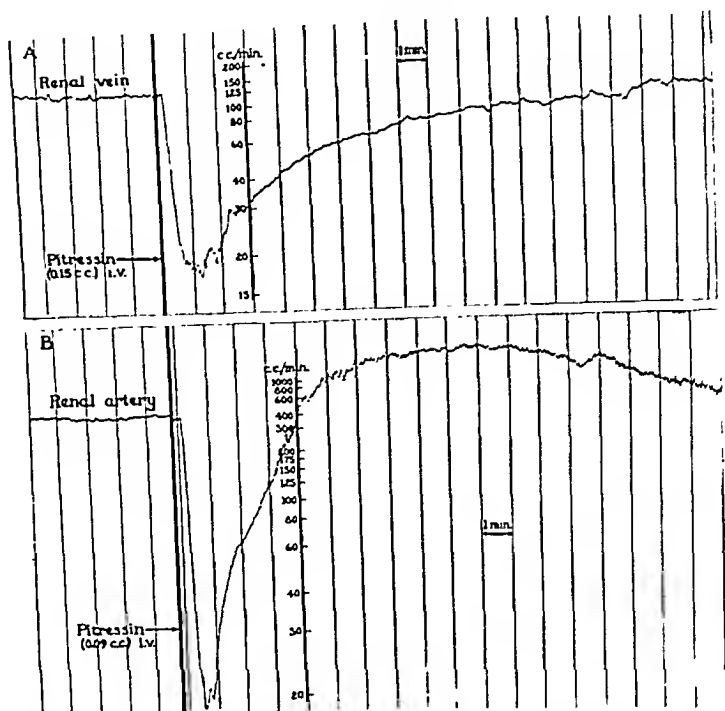


Fig. 1.—Effect of pitressin on the renal blood flow in the trained dog. *A* demonstrates the sudden and marked decrease of blood flow in the renal vein under the influence of intravenously administered pitressin. More than eighteen minutes elapsed before the blood flow returned to the preinjection level. *B* demonstrates the effect of a small dose of pitressin given intravenously on the blood flow in the renal artery. This was markedly decreased, but within five minutes the flow increased beyond the preinjection level.

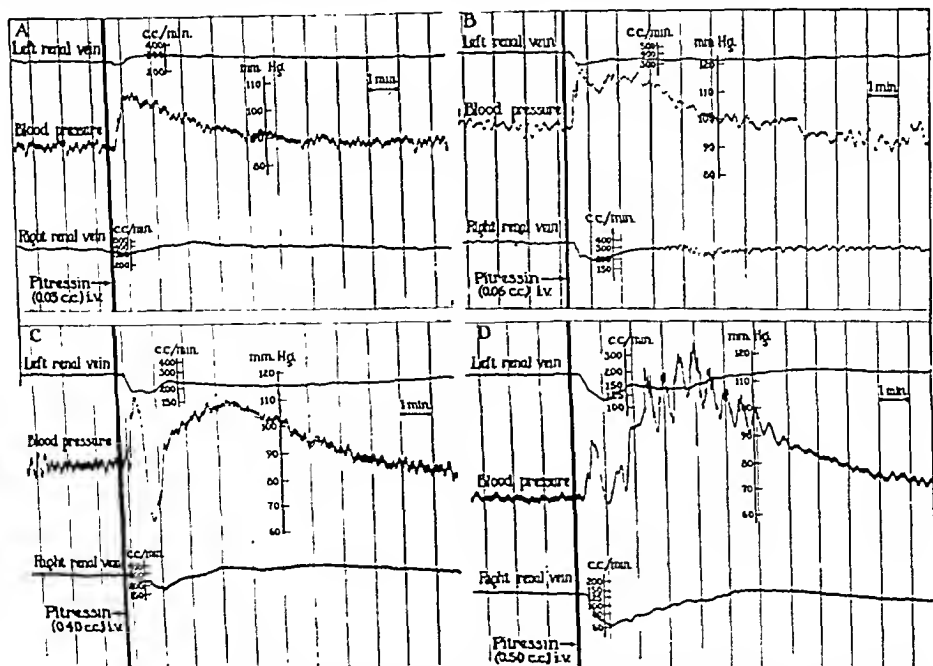


Fig. 2.—Effect of various doses of pitressin on the carotid blood pressure and on the blood flow in the right and left renal veins of the anesthetized animal (pentobarbital sodium).

Three groups of healthy dogs were used for investigating this phase of the problem. The anesthetic used on the first group was pentobarbital sodium, which was slowly administered intravenously in amounts of 25 mg. per kilogram of body weight. The kidney was well exposed through a midline incision of the abdomen, and the renal artery or vein was gently dissected and carefully cleaned, avoiding injury to any of the nerves in the hilus. A calibrated unit was snugly fitted around the renal vessel and conveniently anchored to avoid any interference or turbulence in the blood flow of the vessel used. The same technique was used in applying another unit on the vessel of the second kidney. In some experiments a unit was placed on the common iliac artery or vein instead of one of the kidney vessels in order to permit simultaneous recording of the effect of pitressin on the blood flow of the kidney as well as of the lower extremity.

TABLE I

PITRESSIN: A COMPARISON OF SUBCUTANEOUS, INTRAMUSCULAR, AND INTRAVENOUS INJECTIONS

| DOG NO.          | WEIGHT (KG.) | OBSERVATIONS MADE                      | DOSE (UNITS PER KG.) | MODE OF INJECTION | PERCENTAGE DECREASE IN RENAL BLOOD FLOW FOLLOWING INJECTION |
|------------------|--------------|--|----------------------|-------------------|---|
| 1                | 13.3         | During pentobarbital sodium anesthesia | 0.6                  | Intravenous       | 66  |
|                  |              |  | 0.6                  | Intramuscular     | 6   |
|                  |              |  | 0.6                  | Intramuscular     | 8   |
|                  |              |  | 0.6                  | Subcutaneous      | 8   |
|                  |              |  | 0.6                  | Subcutaneous      | 8   |
|                  |              |  | 0.6                  | Intravenous       | 75  |
| 2                | 19.3         | During chloralosan anesthesia          | 0.6                  | Intravenous       | 48  |
|                  |              |  | 0.6                  | Intramuscular     | 3   |
|                  |              |  | 0.6                  | Subcutaneous      | 1   |
|                  |              |  | 0.6                  | Subcutaneous      | 9   |
|                  |              |  | 0.6                  | Intramuscular     | 7   |
|                  |              |  | 0.6                  | Intravenous       | 7   |
| 3<br>Trained dog | 17           | Second day after operation             | 0.3                  | Subcutaneous      | 7   |
|                  |              |  | 0.075                | Intravenous       | 38  |
|                  |              |  | 0.3                  | Intramuscular     | 10 (increase)*  |
|                  |              |  | 0.15                 | Intravenous       | 47  |
|                  |              | Third day after operation              | 0.15                 | Intravenous       | 55  |
|                  |              |  | 0.45                 | Intramuscular     | 11 (increase)*  |
| 4<br>Trained dog | 21.7         | Second day after operation             | 0.30                 | Intravenous       | 51  |
|                  |              |  | 0.30                 | Intravenous       | 96  |
|                  |              | Third day after operation              | 0.15                 | Intravenous       | 96  |
|                  |              |  | 0.075                | Intravenous       | 96  |
|                  |              |  | 0.3                  | Intramuscular     | 30  |
|                  |              |  | 0.3                  | Subcutaneous      | 21  |
|                  |              |  | 0.3                  | Subcutaneous      | 21  |

\*In two instances the flow was increased slightly by intramuscular injections in Dog 3.

NOTE: Intravenously administered, pitressin produces consistently a decrease in renal blood flow; intramuscularly, it gives variable results; subcutaneously, it usually has no effect on the renal blood flow.

Both ureters were isolated and cannulated at their entrance into the bladder. Y-shaped glass tubing was connected to the ureteral cannulas to lead the urine into a common exit for recording the rate of urine flow. The femoral vein was exposed for intravenous administration, and the carotid artery was cannulated for recording blood pressure. Heparin was used as anticoagulant in the system for optical recording of the carotid blood pressure. The abdomen was closed, leaving between the sutures the tubing of the ureteral cannulas and the flexible, rubber-insulated lead wires of the units. The two galvanometer deflections translating the blood flows in the two kidneys and the optical record of



the blood pressure were all photographed on slowly moving photosensitive paper, thus furnishing permanent records of control values, and of any changes during and after the administration of pitressin for any length of time desired.

The same technical procedures and observations mentioned in the preceding paragraph were made on another group of dogs which were anesthetized by slow intravenous administration of chloralosan—100 mg. per kilogram of body weight, dissolved in warm Ringer's solution.

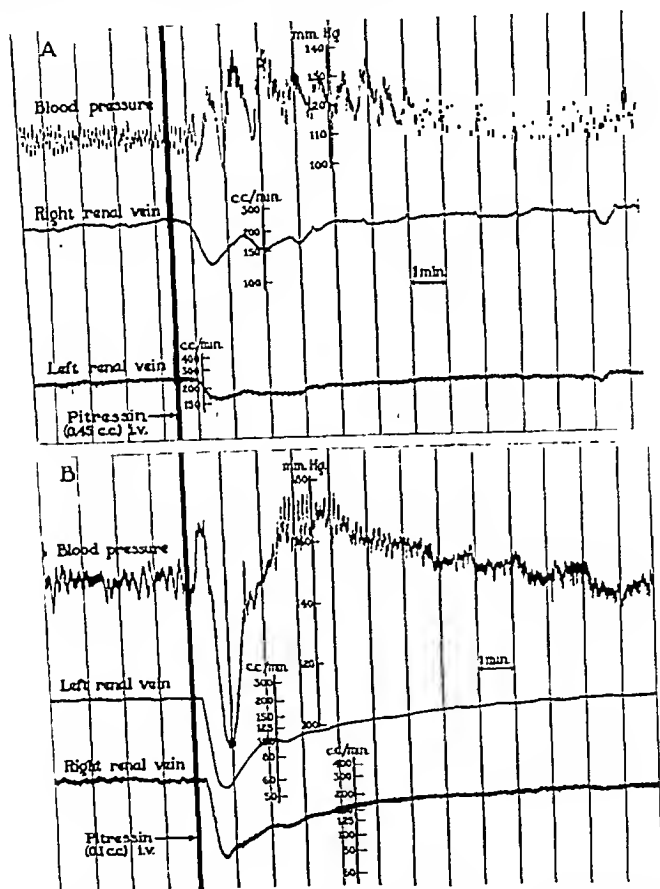


Fig. 3.—Effect of pitressin under different anesthetics: *A*, chloralosan; *B*, pentobarbital sodium. Under chloralosan, the injection of pitressin, in spite of the much larger dose, gave a much smaller decrease in renal blood flow than did pentobarbital sodium.

For the purpose of avoiding the influence of anesthesia, another series of experiments were performed on dogs that had been trained to lie quietly on the table. On a day prior to the experiment, the dogs were etherized and, with the usual aseptic technique, the renal vessel was exposed and a calibrated unit was applied as previously described. The lead wires of the unit were exteriorized, and the abdominal incision was carefully sutured according to surgical principles. Observations on renal blood flow were made after the dog had recovered from the operation. After a few days of blood flow studies, the femoral artery was exposed under local anesthesia, and the blood pressure was optically recorded simultaneously with the renal blood flow on the same chart.

TABLE II

EFFECT OF SUCCESSIVE DOSES OF PITRESSIN (GIVEN INTRAVENOUSLY) ON BLOOD FLOW IN RENAL VEINS (WEIGHT OF DOG, 13.2 KG.)

| DOSE (UNITS PER KG.) | PERCENTAGE DECREASE IN BLOOD FLOW |                  |
|----------------------|-----------------------------------|------------------|
|                      | LEFT RENAL VEIN                   | RIGHT RENAL VEIN |
| 0.15                 | 67                                | 70               |
| 0.15                 | 66                                | 58               |
| 0.15                 | 75                                | 72               |
| 0.15                 | 63                                | 65               |
| 0.075                | 50                                | 49               |
| 0.075                | 54                                | 57               |

TABLE III

EFFECT OF PITRESSIN ON URINE FORMATION IN ANESTHETIZED DOGS

| DOG NO. | WEIGHT (KG.) | DOSE (UNITS PER KG.) | MODE OF INJECTION | URINE, DROPS PER MINUTE |                 | DURATION OF EFFECT (MINUTES) |
|---------|--------------|----------------------|-------------------|-------------------------|-----------------|------------------------------|
|         |              |                      |                   | BEFORE INJECTION        | AFTER INJECTION |                              |
| 1       | 13.3         | 0.6                  | Intravenous       | 6                       | 3               | 6                            |
|         |              | 0.6                  | Intramuscular     | 4                       | 4               | No effect                    |
|         |              | 0.6                  | Subcutaneous      | 6                       | 6               | No effect                    |
| 2       | 19.3         | 0.6                  | Intravenous       | 6                       | 1               | 9                            |
|         |              | 0.6                  | Intramuscular     | 13                      | 9               | 30                           |
|         |              | 0.6                  | Subcutaneous      | 12                      | 12              | No effect                    |
| 5       | 13.8         | 0.4                  | Intravenous       | 3                       | 1               | 8                            |
|         |              | 0.4                  | Intramuscular     | 2                       | 2               | No effect                    |
|         |              | 0.4                  | Subcutaneous      | 1                       | 1               | No effect                    |
|         |              | 0.4                  | Subcutaneous      | 1                       | 1               | No effect                    |
| 6       | 12.0         | 0.5                  | Intravenous       | 5                       | 1               | 8                            |
|         |              | 0.5                  | Intramuscular     | 3                       | 2               | 60                           |
| 7       | 18.8         | 0.3                  | Intravenous       | 4                       | 1               | 7                            |
|         |              | 0.5                  | Intramuscular     | 11                      | 10              | 60                           |
| 8       | 22.0         | 0.65                 | Intravenous       | 4                       | 1               | 8                            |
|         |              | 0.65                 | Intramuscular     | 3                       | 3               | No effect                    |
| 9       | 15.4         | 0.5                  | Intravenous       | 5                       | 1               | 10                           |
|         |              | 0.5                  | Intramuscular     | 3                       | 3               | No effect                    |

NOTE: Intravenous administration of pitressin immediately causes anuria followed by oliguria for about six to ten minutes. Intramuscularly it produces slight oliguria, but subcutaneously it does not have any effect on the urine formation in the anesthetized animal.

**Results.**—When given intravenously, pitressin has a pronounced effect on the renal blood flow in the trained dog, that is, a marked decrease (Fig. 1). This effect depends on the amount of the drug injected (Fig. 2). The decrease in flow is less pronounced when the dog is anesthetized and seems to vary with the kind of anesthetic used (Fig. 3).

When pitressin is given either intramuscularly or subcutaneously, its effect is less marked. In contrast to the intravenous injection, the subcutaneous injection produces no significant change in either the renal blood flow or in the systemic blood pressure. Intramuscular injections produce slight but variable changes in renal flow (Table I).

Some investigators have expressed the opinion that subsequent injections of pitressin do not have as marked an effect as the first injection. We have not been able to confirm this. Successive injections were never made, however, until the renal blood flow had returned to the preinjection value. Under these circumstances successive injections caused a decrease similar to that of the first (Table II).

Concerning the effect of pitressin on blood pressure, we wish to call attention to the fall in blood pressure following the immediate transient rise (Figs. 2C and 3B) which sometimes takes place previous to the prolonged moderate increase.

The decrease in renal blood flow is not maintained over as long a period as that in the femoral or carotid arteries. Another distinguishing characteristic is that the initial decrease in renal flow is followed sometimes by a prolonged and marked increase in the trained dog (Fig. 1B).

The well-known decrease in urine flow occurred following intravenous injections of pitressin (Table III).

#### SUMMARY

By means of transillumination, direct observations were made on the effect of pitressin on the glomerular tuft of capillaries and other vessels in the frog kidney. Various dilutions of pitressin were applied directly to the illuminated area of the kidney, or were injected into the lymph sac or into the abdominal vein of the frog under urethane anesthesia. Depending on the amount and dilution used, pitressin produced cessation of the circulation in the glomerular tuft of capillaries for a fraction of a minute to several minutes, and slowed the circulation in the other vessels to such an extent that no axial stream was observed, and the white and red corpuscles could be easily distinguished in the slowly moving stream within the lumen of the vessel.

Simultaneous records of urine and renal blood flows and optical records of blood pressure were made on a group of dogs under pentobarbital sodium anesthesia and on another group under chloralosan anesthesia. It was observed that pitressin, subcutaneously administered, caused no changes in urine or renal blood flows or in the blood pressure. Intramuscular injections produced variable but slight changes; but, when given intravenously, pitressin always produced transient anuria followed by oliguria for several minutes and a marked decrease in renal blood flow, from which recovery usually was not complete in half an hour, and sometimes not even in one hour. Intravenous injections of pitressin produced a sudden but transient rise in blood pressure, followed occasionally by a transient fall, and then by a gradual prolonged but moderate rise which lasted for approximately a quarter hour.

A series of observations on renal blood flow was performed on a group of trained dogs. The results under influence of pitressin injected subcutaneously, intramuscularly, or intravenously were practically the same as in the anesthetized groups. However, it was observed that the decrease in renal blood flow after intravenous administration of pitressin was more marked and usually lasted longer in trained dogs than in anesthetized dogs.

#### CONCLUSIONS

The data presented justify the conclusion that the same preparation of pitressin administered under identical conditions into the same animal produces different effects on the renal blood flow, depending primarily on the route of administration. Subcutaneously administered, it produces very slight, if any, change in the blood flow. Intramuscular injections give variable results, pos-

sibly dependent on the vascularity and activity of the area involved and its influence on the rate of absorption. Intravenously administered pitressin invariably decreases the blood flow, and caution regarding its intravenous use may aid in the prevention of untoward effects.

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## SILICON IN NONSILICOTIC LUNGS: ITS RELATION TO APICAL SCARS AND TO NODULES\*

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IT HAS been accepted generally that the fibrotic scarring observed at the apices of lungs is of tuberculous origin. This view has been held until recently, when it was suggested that carbon pigment plays a part in the development of such scars. Still more recently, work has been done to demonstrate the relationship between the accumulation of silicious dust in the lungs and the presence of scars showing no evidence of healed tuberculosis.

### ETIOLOGIC CONCEPTION OF APICAL SCARS

*Tuberculosis.*—In 1900 Naegeli<sup>1</sup> reported a study of the incidence of tuberculosis in 500 cases in which necropsy was performed. Apical scars were present in the lungs in 90 per cent of the cases in which the patients had lived in cities and had died of causes other than tuberculosis. He, therefore, suggested that the scars were of tuberculous origin. His view has been supported by many other workers. Aschoff,<sup>2</sup> in particular, stated that 90 per cent of adults have apical scars and affirmed that all of these are of tuberculous origin. Few investigators have put forth a satisfactory explanation as to why this scarring occurs. Opie<sup>3</sup> expressed the opinion that the lesion is a secondary type of infection derived from an exogenous source. He found apical scars in one of five or six adults in the later decades of life. He also found that many lesions were active and that the primary complex was calcified. Consequently, he expressed the opinion that this lesion is a secondary type of infection. Van Zwaluwenburg and Grabfield<sup>4</sup> in 1921 were convinced that this lesion is closely associated with a previous tuberculous infection of the tonsils or cervical lymph nodes. Grober<sup>5</sup> injected India ink into the pharynx of dogs. The dogs were killed and the lymphatics of the neck and apical regions were dissected to determine the dissemination of the ink. He found that the ink had been carried down the cervical lymphatics directly into the pleurae of the apices of the lungs. He concluded that apical scarring is brought about in this manner and that this is possibly the route of infection in many cases of pulmonary tuberculosis. Baldwin, and co-workers<sup>6</sup> of the Trudeau Foundation, expressed the opinion that the infection reaches the apex through the blood stream by way of the pulmonary artery. They subscribed to the hypothesis that the hilus node in the active stages of the primary infection is unable to hold all the organisms of tuberculosis that lodge there, and consequently the organisms are thrown off into the thoracic duct and thus into the pulmonary artery.

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*Dust.*—While relatively little work has been done concerning the possibility of inhaled dusts as an etiologic agent for apical scars, what has been done seems to indicate a silicotic or dust origin for these scars contrary to the view of others that they are invariably of tuberculous origin. The work does not rule out, however, other inflammatory processes. Histologic examination of the lungs often shows an excess of anthracotic deposits associated with various degrees of fibrosis; and since apical scars are frequently associated with heavy anthracotic deposits, some workers have suggested that carbon pigment may play a part in the development of the scars, although at the same time they feel that the lesions are tuberculous in origin. This view has been expressed by Sato.<sup>7</sup> Davson and Susman<sup>8</sup> in 1937, studying micro-incinerated sections, concluded that the development of apical scars is secondary to the accumulation of silicious matter at the apex of the lungs. Also they showed that there is a definite relationship between silicious dust accumulating in the upper part of the lung and the scars that did not show evidence of tuberculosis.

#### MATERIAL AND METHODS

*Micro-incineration.*—Without going into the details of the technique of micro-incineration, let it suffice to say that it is a histologic method to demonstrate the presence and distribution of some of the various mineral components in tissues. The method is based upon the principle that, when a section of tissue is subjected to a temperature of 600° C., all organic material is destroyed, leaving the inorganic ashes. In these ashes are found the heat-resistant minerals, and in particular in the case of lung tissues, the deposits of the silicon compounds. The method is of value in determining the relative quantity of silicious matter in a given section of lung tissue and is of particular value in differentiating the silicotic nodule from the tuberculous nodule, as shown by Policard and Doubrow,<sup>9</sup> and Irwin<sup>10</sup> (Figs. 1, 2, 3, and 4). In the silicotic nodule the silicious ash is distributed rather uniformly throughout the entire nodule, while in the tuberculous nodule the silicious material is limited to the periphery of the nodule in the region corresponding to the capsule. Irwin inferred that if the ash pattern of silicosis is found, it represents a silicotic nodule, even if the corresponding area in the stained section has degenerated beyond recognition; and if such an area does not contain any particles of ash or only a few scattered ones, it is an area of nonsilicious fibrosis.

*Method of Procedure for This Study.*—The lungs in 200 patients in whom necropsy was performed at the Mayo Clinic from Sept. 1, 1938, to Dec. 31, 1938, inclusive, were examined. On gross inspection, the degree of anthracosis and the grade of apical scarring were noted. Both findings were recorded on a basis of 1 to 4. In addition, the presence or absence of evidence of healed tuberculosis in the lung or hilus lymph nodes was observed. The lungs that did not show evidence of healed tuberculosis were examined roentgenographically by Squire<sup>11</sup> for calcium deposits which were later recovered by him. Cases in which calcium deposits were found in the lungs were added to those cases of healed tuberculosis already ascertained by visual inspection. Finally, the age, sex, occupation, and residence of the deceased were recorded. The great majority of these 200 persons were adults more than 20 years of age,

while the minority consisted of younger persons, including a few children. Also, the lungs and portions of lungs in several known cases of silicosis were examined. A similar examination was made of the lungs of a few newborn infants. The latter two sets of lungs, those from persons who had had silicosis

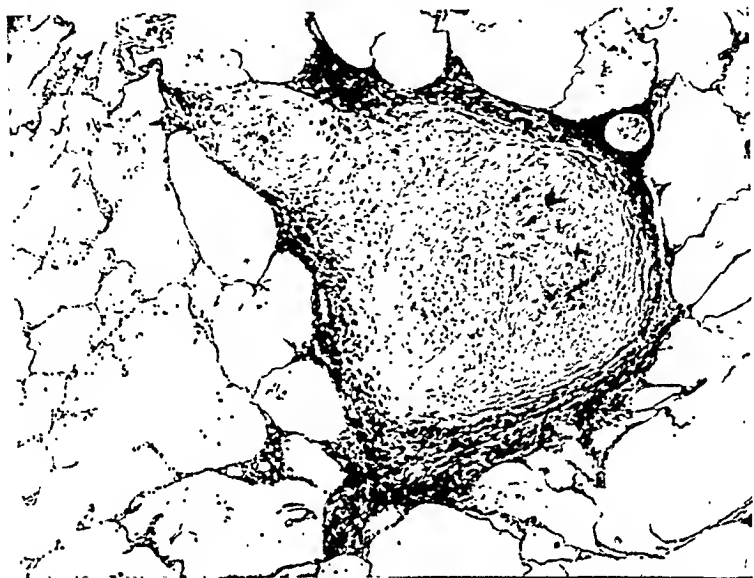


Fig. 1.—Silicotic nodule in a known case of silicotuberculosis (hematoxylin and eosin,  $\times 42$ ).



Fig. 2.—Consecutive section to Fig. 1, incinerated, showing ash pattern of a silicotic nodule (dark field,  $\times 42$ ).

and those from infants, were examined for the purpose of comparison and control, but neither of these sets was included in the 200 afore-mentioned cases, none of which revealed any evidence of clinical silicosis during life.

The lungs were sectioned longitudinally at intervals of  $\frac{1}{2}$  inch (1.3 cm.) to determine whether or not there was any gross evidence of silicosis, such as the presence of a nodule in the lung or hilus nodes, the presence of "egg shell"

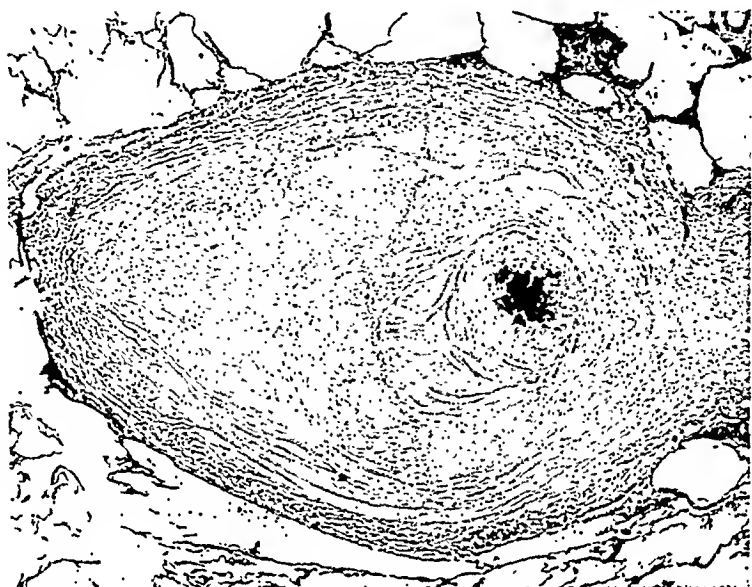


Fig. 3.—Tuberculous nodule situated near the silicotic nodule shown in Fig. 1 (hematoxylin and eosin,  $\times 34$ ).

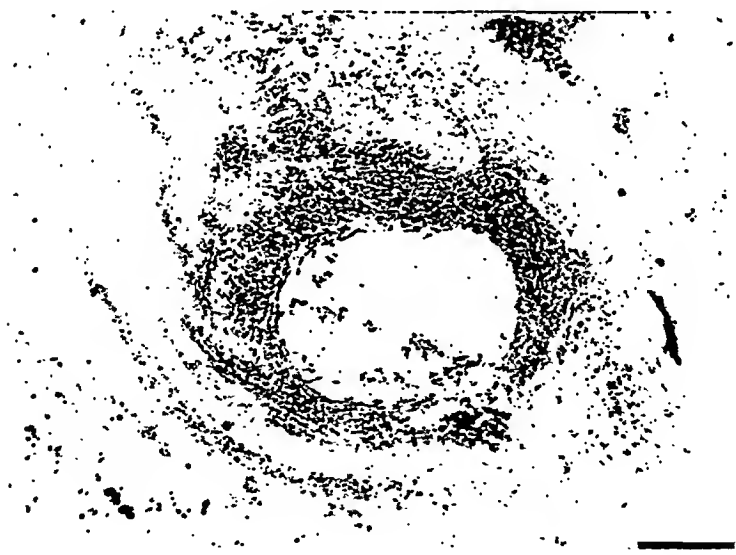


Fig. 4.—Consecutive section to Fig. 3, incinerated, showing ash pattern of a tuberculous nodule (dark field,  $\times 34$ ).

calcification in the hilus nodes, or the presence of suspicious regions of fibrosis. If any such lesions were discovered, blocks of tissue were made of the region involved and were placed in a fixative of 10 per cent formalin. Then routinely one block of tissue was cut from the apical scar or from the apex in those lungs



not revealing a scar, one block from the upper lobe near the scar but not including it, one block from the lower lobe, and one of a hilus lymph node that did not contain calcareous material. These blocks, after fixing, were embedded in paraffin in the usual manner, and serial sections were cut of a thickness of 5 microns. Three sections were cut, one for staining with hematoxylin and eosin, and two unstained sections for micro-incineration. After incineration the sections were treated with concentrated hydrochloric acid so as to remove the inorganic ash, and then were examined with dark-field illumination. By comparison with the consecutive stained sections, an exact localization of any deposits of silicious ash was obtained. Furthermore, since all sections were of equal thickness, the amount of silicious ash was compared by visual estimation.

For the purpose of estimation of the amount of silica in any given section, four grades were recognized: grade 1, slight amount of ash deposits about the blood vessels or bronchioles; grade 2, moderate amount of ash deposits about the blood vessels or bronchioles associated with a slight amount of diffuse scatterings of ash throughout the parenchyma; grade 3, large collections of ash deposits about vessels and bronchioles associated with a uniform scattering of ash throughout the parenchyma; and grade 4, large collections of local deposits associated with large deposits throughout the parenchyma. In the case of silica grade 4, the amount corresponded to that found in sections from the fibrotic portions of silicotic lungs.

#### RESULTS

The apical scar lesion occurred at the apex of either one or both lungs and was characterized by a firm, grayish-white, opaque, "scarred" region with variable degrees of puckering of the visceral pleura. In some cases the scarring was more extensive and formed a cap. A few of these caps felt on palpation as if they were constructed of cartilage. Sections of the region had in some cases a dark anthracotic fibrosis in the subjacent lung substance without any evidence of caseation or calcification. A few scars revealed gross evidence of calcification. In some cases the parenchyma of the lung directly beneath the scar contained an emphysematous cavity or multiple smaller ones.

By histologic examination there was fibrous thickening of varying depth, and the subjacent lung parenchyma revealed a greater or lesser degree of fibrosis. The fibrous tissue when stained with hematoxylin and eosin usually exhibited a bluish pink to pink color of the connective tissue fibers. In many cases the connective tissue had become hyalinized. The extent of the fibrotic regions varied; in younger people the lesions were smaller, while in the older age groups the more massive lesions usually occurred. There was considerable proliferation of collagen fibers throughout the fibrotic regions in some of the lesions, some of which could be traced into the parenchyma of the adjacent lung and merged with it. The fibrotic regions contained varying amounts of carbon pigment scattered diffusely throughout the scar. In some cases numerous alveoli containing clumps of phagocytes laden with carbon occurred within the fibrotic regions. In four scars giant cells were seen.

Micro-incineration of the apical scars showed that the deposits of silica were always present with the carbon and in corresponding amounts, except for

a few cases in which the siliceous matter was greater than the corresponding carbon and vice versa (Figs. 5, 6, 7, and 8). Also as far as the lungs were concerned, the siliceous material was found to be more or less uniform throughout, although there was a moderate increase in the silica grade in the upper

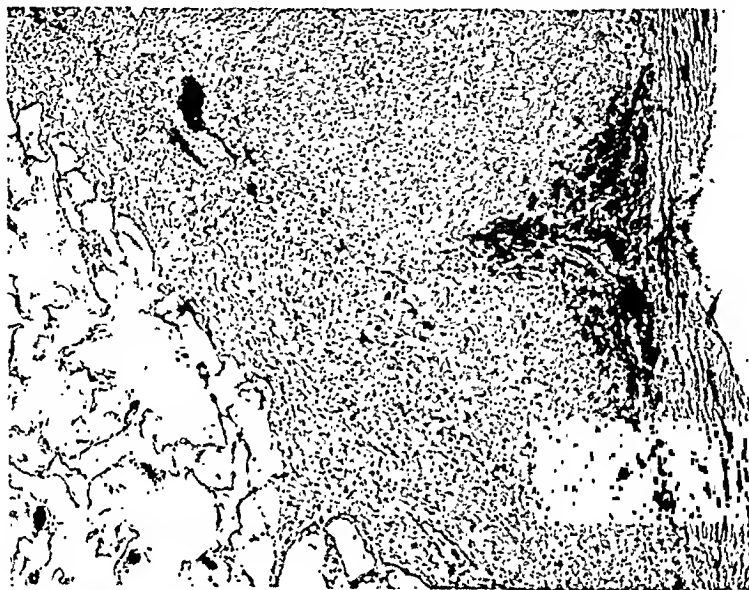


Fig. 5.—“Nonspecific” apical scar (hematoxylin and eosin,  $\times 42$ ).

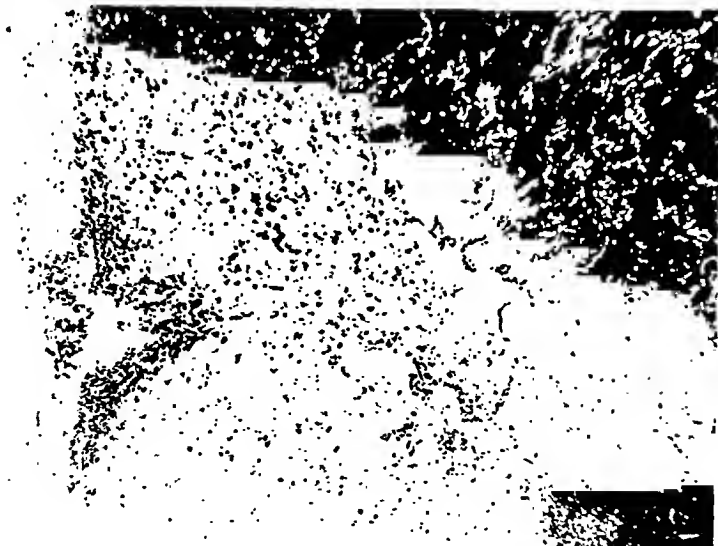


Fig. 6.—Consecutive section to Fig. 5, incinerated, showing diffuse and local collections of siliceous deposits in precisely the same locations as the carbon deposits (dark field,  $\times 42$ ).

lobes. In a few apical scars and in many lungs and hilus lymph nodes the intensity of silica was quite comparable to that seen in microscopic sections from silicotic lungs. In these cases there were seen in the stained section areas of fibrosis characterized by circumscribed hyalinized areas which appeared to

be identical with silicotic nodules. They contained the finely distributed carbon sprinkling commonly seen in silicotic nodules. Incineration of these lesions revealed that they contained the silica ash pattern appearing very similar to those found in known silicotic nodules. In some of the better incinerated

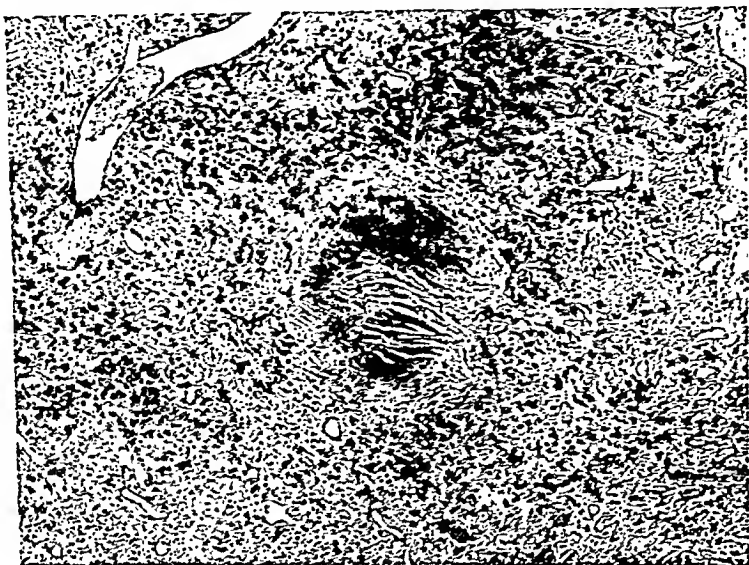


Fig. 7.—“Specific silicotic” or dust “apical scar.” Note silicotic nodule in the apical scar (hematoxylin and eosin,  $\times 48$ ).

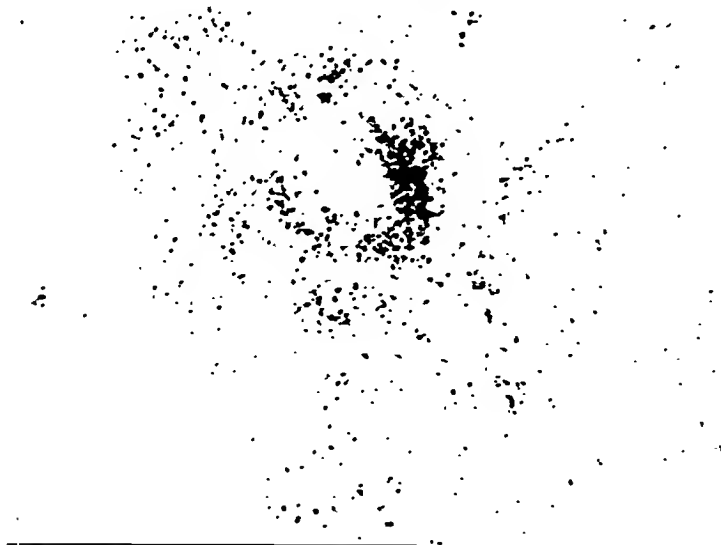


Fig. 8.—Consecutive incinerated section to Fig. 7 (dark field,  $\times 48$ ).

sections of apical scars there was noted the ash pattern of the scar, and in the background appeared a faint ash network which somewhat resembled the architecture of lung tissue and which merged with the ash pattern of the normal adjacent lung. This ash pattern seen in the background of the fibrotic

areas was no doubt the lung that previously had occupied the area and which was replaced by collagenous connective tissue.

The findings of this study are given in seven different tables. Table I summarizes the number of cases of apical scars in both sexes and their relation to the age of the patients and to the amount of anthracosis in their lungs. There was no anthracosis in the first decade of life; anthracosis grade 1 began in the second decade, with a maximal number of cases in the seventh decade; anthracosis grade 2 began in the third decade, with a maximum in the sixth decade; anthracosis grade 3 began in the fifth decade, with a maximum in the seventh decade; and anthracosis grade 4 occurred only in the seventh decade. Of the 133 cases of apical scars (66.5 per cent) the least number occurred in the third and ninth decades, and the greatest number in the seventh decade. Cases of apical scarring did not occur during the first two decades. Finally, it is noted that the degree of anthracosis increased with the age and the number of cases of apical scars increased with age as the grade of anthracosis increased, this being true up to the higher decades of life, at which point the number decreased.

TABLE I

GRADE OF ANTHRACOSIS AND OCCURRENCE OF APICAL SCARS BY AGE OF PATIENTS, BOTH SEXES

| AGE (YR.)   | CASES | GRADE OF ANTHRACOSIS |     |    |    |   | APICAL SCARS |          |
|-------------|-------|----------------------|-----|----|----|---|--------------|----------|
|             |       | 0                    | 1   | 2  | 3  | 4 | CASES        | PER CENT |
| 0-9         | 2     | 2                    | 0   | 0  | 0  | 0 | 0            | 0.0      |
| 10-19       | 10    | 3                    | 7   | 0  | 0  | 0 | 0            | 0.0      |
| 20-29       | 10    | 0                    | 9   | 1  | 0  | 0 | 6            | 60.0     |
| 30-39       | 14    | 0                    | 13  | 1  | 0  | 0 | 8            | 57.0     |
| 40-49       | 28    | 0                    | 20  | 7  | 1  | 0 | 18           | 64.0     |
| 50-59       | 46    | 0                    | 30  | 14 | 2  | 0 | 35           | 76.0     |
| 60-69       | 54    | 0                    | 40  | 8  | 4  | 2 | 39           | 72.0     |
| 70-79       | 25    | 0                    | 15  | 8  | 2  | 0 | 21           | 84.0     |
| 80 and more | 11    | 0                    | 7   | 3  | 1  | 0 | 6            | 54.0     |
| Total       | 200   | 5                    | 141 | 42 | 10 | 2 | 133          | 66.5     |

TABLE II

AVERAGE AGE OF PATIENTS AND OCCURRENCE OF APICAL SCARS BY GRADE OF ANTHRACOSIS

| GRADE OF ANTHRACOSIS | SEX  |        | TOTAL CASES | AVERAGE AGE (YR.) | APICAL SCARS |          |
|----------------------|------|--------|-------------|-------------------|--------------|----------|
|                      | MALE | FEMALE |             |                   | CASES        | PER CENT |
| 0                    | 2    | 3      | 5           | 8.6               | 0            | 00.0     |
| 1                    | 81   | 60     | 141         | 54.5              | 89           | 63.1     |
| 2                    | 35   | 7      | 42          | 58.5              | 34           | 81.0     |
| 3                    | 10   | 0      | 10          | 65.0              | 8            | 80.0     |
| 4                    | 2    | 0      | 2           | 66.5              | 2            | 100.0    |
| Total                | 130  | 70     | 200         |                   | 133          | 66.5     |

While Table I reveals the relationship of apical scars and anthracosis to the various age groups, a much better relation is obtained by studying Table II, which shows the relationship of anthracosis and apical scars to the average age. Here it is noted that the majority of cases are anthracosis grade 1, and that the number of cases decreases as the grade of anthracosis increases beyond grade 1, but that the average age of the patients increases. The percentage of apical scars increases with the average age and with the grade of anthracosis.

Thus far, the discussion has been concerned mostly with anthracosis. The reason for this will be obvious when Table III is studied. This table, con-

structured exactly like Table II, shows the relationship of apical scars to the average age and to the total deposits of silica in the lungs, as estimated by micro-incineration. Here it is noted that of the 200 cases studied, 157 reveal silica grade 1 in the lungs, while a decreasing number of cases reveal silica, grades 2, 3, and 4, respectively. Ten cases reveal no silica at all, and the average age of this group is 24 years. Of the cases of silica grade 1, the average age is 54.3 years. Of the grades of silica higher than 1, the average age increases for each silica grade group, except grade 4, while the total number of cases for each silica grade decreases beyond the peak of silica grade 1. As concerns the apical scars, here again it is noted that the percentage of apical scars increases with age as the total content of silica of the lungs increases. After studying Tables II and III, one is struck by the close parallelism of the gross anthracotic grade with the microscopic total silica grade with respect to the total number of cases, the average age, and the percentage of apical scars. Thus far, the study seems to verify the findings of previous workers, namely, that silica deposits occur in conjunction with anthracotic deposits, and in roughly corresponding amounts. In addition, the findings suggest that the amount of anthracosis determined by gross examination of the lungs is a fairly good index to the total amount of silicious material present in the same lungs.

TABLE III

AVERAGE AGE OF PATIENTS AND OCCURRENCE OF APICAL SCARS BY GRADE OF TOTAL SILICA IN LUNGS AS ESTIMATED BY MICRO-INCINERATION

| GRADE OF<br>TOTAL SILICA | SEX  |        | TOTAL<br>CASES | AVERAGE<br>AGE (YR.) | APICAL SCARS |          |
|--------------------------|------|--------|----------------|----------------------|--------------|----------|
|                          | MALE | FEMALE |                |                      | CASES        | PER CENT |
| 0                        | 6    | 4      | 10             | 24.0                 | 2            | 20.0     |
| 1                        | 95   | 62     | 157            | 54.3                 | 104          | 66.2     |
| 2                        | 25   | 4      | 29             | 64.3                 | 23           | 79.3     |
| 3                        | 3    | 0      | 3              | 69.6                 | 3            | 100.0    |
| 4                        | 1    | 0      | 1              | 68.0                 | 1            | 100.0    |
| Total                    | 130  | 70     | 200            |                      | 133          | 66.5     |

What has been said concerns the apical scar and its relation to the silica deposits in the lungs. Now what about the relation of this lesion to the silica deposits in the scar itself, and the relation of the latter to the silica deposits in the respective lungs and hilus lymph nodes? It is noted in Table IV that as the thickness of the apical scar increases, the total content of silica in the apical scars and their respective lungs and hilus nodes tends to increase. This is much more evident in the male sex.

Of the 133 cases of apical scars, it was of interest to note that in only four cases (3.0 per cent) and possibly in only 3 cases, was there no evidence of tuberculosis in the lungs or hilus lymph nodes. In other words, in 129 cases of apical scars (97.0 per cent) there were signs of tuberculosis in either the lung or the hilus lymph nodes. Of the 4 nontuberculous patients, 2 were men and 2 were women. One patient was a 73-year-old man with a very thick apical scar and a correspondingly high silica content in the scar, lung, and hilus nodes, while the other 3 cases were in young persons (ages 24, 30, and 32 years) with correspondingly low silica determinations. While it is possible for an old man not to have evidence of healed pulmonary tuberculosis, it must be stated that this was the only case in which roentgenologic examination of the lungs for calcium deposits

was not made. Hence the previous statement that possibly in only 3 cases was there no evidence of tuberculosis in the lungs or hilus nodes is probably correct.

TABLE IV  
APICAL SCARS WITH RELATION TO THEIR SILICA CONTENT

| SEX    | GRADE OF SCAR | CASES | TOTAL SILICA, AVERAGE GRADE ON BASIS OF 1 TO 4 |      |            |
|--------|---------------|-------|--|------|------------|
|        |               |       | SCAR   | LUNG | HILUS NODE |
| Male   | 1             | 47    | 1.4  | 1.3  | 2.2        |
|        | 2             | 26    | 1.7  | 1.3  | 2.4        |
|        | 3             | 10    | 2.1  | 1.9  | 2.6        |
|        | 4             | 5     | 2.0  | 1.2  | 2.2        |
| Female | 1             | 28    | 1.1  | 0.9  | 2.3        |
|        | 2             | 12    | 1.6  | 1.3  | 2.3        |
|        | 3             | 4     | 1.6  | 1.2  | 2.3        |
|        | 4             | 1     | 1.0  | 0.7  | 2.0        |
| Total  |               | 133   |  |      |            |

Of the 67 cases without apical scars, there was evidence of tuberculosis in the lungs or hilus nodes in 48 (72 per cent) (Table V). The average total content of silica in the lungs and hilus nodes of the men without apical scars, but with evidence of tuberculosis, is found to be somewhat less than for the men with apical scars. As to the female sex, the corresponding silica determinations are considerably less than for those females having apical scars.

TABLE V  
PATIENTS WITH NO APICAL SCARS WITH TUBERCULOSIS PRESENT IN LUNGS OR HILUS NODES

| SEX    | CASES | AGE, YEARS |          |        | TOTAL SILICA, AVERAGE GRADE |            |
|--------|-------|------------|----------|--------|-----------------------------|------------|
|        |       | MEAN       | YOUNGEST | OLDEST | LUNG                        | HILUS NODE |
| Male   | 36    | 54.5       | 20       | 97     | 1.2                         | 2.2        |
| Female | 12    | 48.3       | 11       | 83     | 0.8                         | 1.8        |

Table VI summarizes the remaining 19 cases, in which there were no apical scars and no evidence of tuberculosis in the lungs or hilus nodes. In general, the patients without evidence of tuberculosis were those in the lower average age groups, and the average total silica was also correspondingly low. On the other hand, the patients that did reveal tuberculosis were in the higher average age groups, and the total content of silica was higher. In nearly all instances the silica content of male patients was somewhat higher than that of female patients.

TABLE VI  
PATIENTS WITH NO APICAL SCARS WITH NO TUBERCULOSIS PRESENT IN LUNGS OR HILUS NODES

| SEX    | CASES | AGE, YEARS |          |        | TOTAL SILICA, AVERAGE GRADE |            |
|--------|-------|------------|----------|--------|-----------------------------|------------|
|        |       | MEAN       | YOUNGEST | OLDEST | LUNG                        | HILUS NODE |
| Male   | 7     | 34.1       | 11       | 68     | 0.6                         | 1.2        |
| Female | 12    | 40.8       | 2        | 76     | 0.7                         | 1.1        |

In passing, a word might be mentioned concerning the general incidence of healed tuberculosis in the 200 cases studied. In 96 cases evidences of healed tuberculosis were found in the lungs by gross dissection. Squire,<sup>11</sup> by post-mortem roentgenologic examination of the lungs, found 28 more cases. Two

cases were found by microscopic examination. This makes a total of 126 cases in which evidences of healed tuberculosis were found in the lungs. In the hilus nodes 127 cases were found by gross dissection. Squire found 38 more cases by roentgenologic examination, making a total of 165 cases in which evidences of healed tuberculosis were found in the hilus nodes. Thus, according to this survey of 200 cases, the incidence of healed tuberculosis is 177 cases, or 88.5 per cent.

*"Silicotic" or "Dust" Nodules in Apical Scars.*—It was previously stated that lesions were found in the lungs or hilus nodes that resembled silicotic nodules and which, upon micro-incineration, revealed the ash pattern of silicotic nodules. In 7 of 133 cases of apical scars, there was an ash pattern comparable to that of known silicotic nodules (Figs. 7 and 8). Thus, the incidence of apical scars that can be said to be "specific silicotic" or "dust scars" is 5.3 per cent. Table VII summarizes these cases. It is noted that in 5 cases the patients were men and in 2 cases they were women, the average age for all being 69 years. The average grade of the apical scars is 2+, and the total silica grade in each scar and hilus node is high, while the silica content of the lung varies. However, note that there is a fairly close correlation between the silica content of the apical scars and the silica content of the hilus nodes.

TABLE VII  
PATIENTS WITH SILICOTIC NODULES IN APICAL SCARS

| SEX     | AGE<br>(YR.) | OCCUPATION           | GRADE OF<br>APICAL<br>SCAR | TOTAL SILICA, GRADE |      |      |
|---------|--------------|----------------------|----------------------------|---------------------|------|------|
|         |              |                      |                            | SCAR                | LUNG | NODE |
| M       | 67           | Street car conductor | 3                          | 4.0                 | 3.0  | 4.0  |
| M       | 55           | Civil engineer       | 2+                         | 2.0                 | 1.0  | 3.0  |
| M       | 77           | Farmer               | 4                          | 3.0                 | 3.0  | 4.0  |
| M       | 71           | Auto trimmer         | 1                          | 3.0                 | 2.0  | 3.0  |
| M       | 73           | Miller               | 2                          | 3.0                 | 1.5  | 3.0  |
| F       | 55           | Housewife            | 2                          | 3.0                 | 1.0  | 4.0  |
| F       | 85           | Housewife            | 2                          | 3.0                 | 1.5  | 4.0  |
| Average | 69           |                      | 2+                         | 3.0                 | 1.9  | 3.6  |

*"Specific Tuberculous" Apical Scars.*—In 4 of the 133 cases of apical scars, there were giant cells, in one case there were tubercles, and in 12 cases there were calcium deposits in the scars themselves. This makes a total of 17 cases of "tuberculous" apical scars (12.8 per cent). In these 17 cases the average age of the patients was 60 years, and the average apical scarring was grade 2+. However, the average silica content of the scar, lung, and hilus node was 1.5, 1.2, and 2.3, respectively, an amount considerably lower than the corresponding average silica content in the "specific silicotic" apical scars.

Since 5.3 per cent of the apical scars are "silicotic" and 12.8 per cent are "tuberculous," the total number of "specific apical scars" is 18.1 per cent. What of the apical scars making up the remaining 81.9 per cent? These apical scars are the "nonspecific scars," characterized by a more or less hyalinized fibrosis of variable thickness, all of which contain some silicious deposits, and all of which, with the exception of 3 or possibly 4 cases, are associated with healed tuberculosis, either in the lungs or hilus lymph nodes or both.

*Incidence of the Silicotic Nodule in the Nonsilicotic Lung.*—By microincineration the ash pattern of the silicotic nodule was found to be present in 21 cases.

Seventeen patients were male and 4 were female with an average age of 68.4 years. The silicotic ash pattern was found in 7 apical scars, in 5 lungs, and in 17 hilus nodes. The finding of the lesion in both the apical scar and the hilus node in the same subject occurred in 2 cases, while the presence of the lesion in the apical scar, in the lung, and in the hilus node in the same subject occurred in 3 cases. The local silica value for all these lesions was high, while the total silica content was relatively high in the respective lungs and hilus nodes. Even the average silica content for all 21 cases was seen to be higher than that found in the great majority of the 200 cases studied. Therefore, on the basis of this study of 200 cases, the incidence of the silicotic nodule in the nonsilicotic lung is 10.5 per cent. In general, the nodules were considerably smaller than the silicotic nodules seen in advanced cases of silicosis. Of all the cases studied, there were none in which the occupation of the patient was predisposed to silicosis; yet there were 21 cases in all of which one or more silicotic nodules were observed. There was little difficulty in distinguishing between healed tuberculous nodules and the nodules due to silica deposits when the method of microincineration was employed. In a few lungs not included in the series of 21 cases, early stages of formation of silicotic nodules were seen in the fibrous capsule of healed tuberculous nodules. It is known that carbon tends to collect around the fibrous walls of such lesions, and in these cases enough silicious material was present, as shown by micro-incineration, to produce the early stages of formation of silicotic nodules.

Considering all cases examined, this study demonstrates that silica deposits are present in normal lungs along with carbon deposits and are coextensive with them. Moreover, the deposits occur in localized regions in quantities comparable with those obtained in cases of diffuse silicosis. Therefore, all persons are exposed to a common source of dust particles. According to Policard and Donbrow, the source might be the smoke from domestic and industrial furnaces, since a high percentage of coal ash is silica. Jephcott<sup>12</sup> quoted figures published by the Chicago Board of Health in 1931, estimating that 134 tons of silicious matter fall per month per square mile. This suggests that all city dwellers during their lifetime inhale appreciable amounts of silica. It is also noteworthy that Campbell<sup>13</sup> found 44 per cent of silica in the dust from tarred roads.

#### COMMENT

By comparing the silica content of the upper lobes with that of the lower lobes it was found that in nearly every case the intensity of carbon and silica accumulations was less in the lower than in the upper lobes. This tendency might be explained by the relative immobility of the apex, as compared with the greater mobility of the lower lobe, so that particles once inhaled tend to lodge in the apical region, while the better ventilation of the rest of the lung tends to favor the removal either by the bronchi or by the lymphatic drainage. Thus, since silicious material tends to collect in the apical region, it is probable that the tendency for scars to occur at the apex is linked to some degree with such dust deposits. This is stated with reserve, because it is quite well known that accumulations of silica occur in regions of fibrosis, regardless of cause. Although the average total silica determined in all the "specific tuberculous" apical scars



is lower than that of the "specific silicotic" scars, there are individual instances in which the silica value is comparable to the dust accumulations found in other apical scars.

### CONCLUSIONS

1. Anthracotic and silicious dust deposits in the lung tissue increase with age, and the grade of anthracosis in a given lung is a rough index to the relative amount of silica deposits present in the same lung, since silica deposits occur in conjunction with anthracotic deposits and in corresponding amounts.

2. The average total content of silica in the lungs of the male is higher than that of the female.

3. The percentage of cases of apical scars increases with age and with the grade of the total silica content of the lung.

4. Of the cases of apical scars 97.0 per cent reveal evidence of healed tuberculosis elsewhere in the lung or hilus lymph nodes.

5. The average total content of silica in lungs possessing apical scars is higher than in those lungs that do not have apical scars.

6. On the basis of 200 cases the incidence of healed tuberculosis in the lungs is 88.5 per cent.

7. On the basis of 133 cases of apical scars the incidence of "specific silicotic" apical scars is 5.3 per cent, and the silica content in these specific cases is relatively high.

8. On the same basis the incidence of the "specific tuberculous" apical scars is 12.8 per cent, and the average silica content in these cases is considerably lower as compared to the "specific silicotic" scars.

9. The incidence of the silicotic nodule in the nonsilicotic lung is 10.5 per cent.

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# FOOD REMNANTS AS A CAUSE OF CONFUSION IN THE DIAGNOSIS OF INTESTINAL PARASITES\*

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## INTRODUCTION

ONE of the everyday problems of the diagnostician in the clinical parasitology laboratory is the differentiation of helminth eggs and protozoan cysts of pathogenic parasites of the intestine, not only from nonpathogenic species and free living organisms, but from various remnants of foods present in feces. The latter, due to their size and general appearance, are often confused with parasites. The object of this study was to find out experimentally what foods are responsible for some of the remains so frequently confused with intestinal parasites.

As early as 1717 van Leeuwenhoek, in examining human exudates, was able to demonstrate that the remains of foods eaten could be recognized in fecal material. The studies initiated by the idea that diseases could be diagnosed by crystals in excreta and the interest aroused in the physiology of digestion in the late nineteenth century led to several works on the composition of the feces. An early worker in this field, Rawitz (1845), classified the residual particles in feces as animal residue, plant residue, single cells, and foreign bodies, but his meaning, particularly in regard to the identity of the "single cells" and "foreign bodies," is not clear. Chiefly concerned with the normal and faulty digestion of foods, much of the work since 1899 has been based upon an extensive monograph by H. L. Q. van Ledden Hülsebosch, who discussed the macroscopic and microscopic structures of numerous plant foods. An excellent summary of the knowledge of fecal constituents was given in 1914 by Cammidge, who recognized the difficulties of gross parasitologic diagnosis, especially of the smaller nematodes and tapeworm proglottids, with which numerous fibers are easily confused upon macroscopic examination. Some anomalies found in microscopic fecal examinations were reported by Faust from China in 1924. Structures which he listed as confusing to the parasitologist were physical artifacts, contaminations, partially digested animal and plant cells, animals introduced by accident, harmless coprozoites, and body cells or tissues produced by noninfective agencies.

## EXPERIMENTAL DATA

In order to secure a point of departure for the study of the origin of some of the confusing remains, it was deemed advisable to find out which of the more common foods were most likely to leave particulate remains resembling

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intestinal parasites in human feces. These undigested or partially undigested food remnants found in feces are difficult, if not at times almost impossible, of identification. However, by becoming acquainted with the structural elements of any given food before it was eaten, it was possible to determine its more characteristic residues. To do this a basic nonresidue diet was used, and for the checking of each individual food studied, a controlled diet was taken. For the remainder of the work hospital patients were used; these patients were on identical diets and their digestion was for all purposes apparently normal.

The basic diet consisted of strained vegetable and fruit juices, ground cooked meat, mashed Irish potatoes, milk, coffee, and white bread. Since cellular structure was absent in the liquids, was destroyed by cooking the bread and potatoes, or was easily recognized, as in the case of the meat, this diet served the purpose well. A small quantity of each food to be studied was obtained in the form in which it was to be served, and was teased apart in saline to separate the various structural elements. Drawings were made of these cells at an average magnification of  $\times 540$ . Stools passed twelve to fifteen hours after the food was eaten were examined for remains of fibers and other structures. These remains were studied, and sketches were made of their characteristic appearance. In both cases the material was studied unstained as well as stained with D'Antoni's iodine.

*Animal Foods.*—Such animal products as milk, cheese, custards, gelatin, and cooked eggs may be classified as nonresidue foods, except when eaten in excessively large amounts, such as milk in the dietary of infants. Of the animal tissues studied, as liver and muscle, only the muscle retained a close resemblance to its original structure, and fecal remnants of muscle fibers could be recognized as such without difficulty. Of the sea foods, crab meat maintained the characteristic structure of arthropod muscle fibers, but oysters and most fish left little residue.

*Plant Foods.*—Undigested plant foods in the form of single cells left the most confusing residues and those most likely to be mistaken for the eggs or cysts of parasites. A variety of common vegetable foods was studied, including representative grains eaten as cereals or in breads, fruits both fresh and dried, leafy vegetables, roots, and some leguminous seeds.

Fragments of whole vegetable tissues were not difficult to recognize as such, for their characteristic cellular arrangements obviate confusion with animal parasites. Bits of the husk of oats and whole corn were often observed to be almost undigested, as well as large pieces of leaf or epidermal tissues and aggregates of fibers from fibrovascular bundles.

Single epidermal cells separated from their tissue did, in a few cases, resemble eggs or cysts, but their heavy cellulose walls and complete lack of internal organization were diagnostic of their plant origin. More confusing were epidermal gland cells or epidermal hairs which, separated from the remainder of the tissue, respectively, resembled tapeworm eggs and small nematode larvae. Palisade cells, particularly from beans or peas, serve as diagnostic landmarks not only for the type of vegetable but also for the particular kinds of legumes, since they are characteristic for each plant, but they are easily differentiated from parasites on the basis of their thick walls and lack of internal structure.

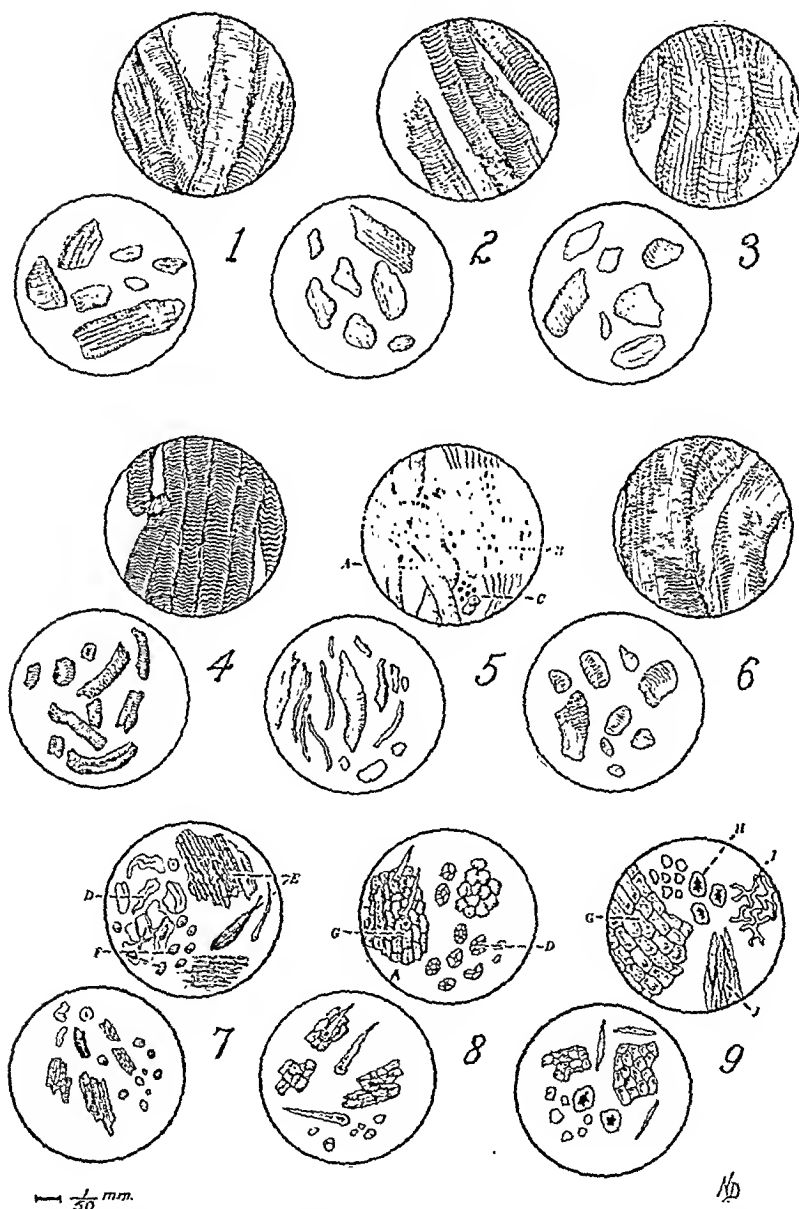


Plate I.—Plates I, II and III represent the characteristic structures of each prepared food, together with their remnants commonly found in feces. The whole foods are figured upward and to the right, remnants are below and to the left of each figure number. 1, Beef muscle; 2, corned beef muscle; 3, pork muscle; 4, crab meat; 5, oyster; 6, fish muscle; 7, wheat; 8, oats; 9, whole kernel corn.

A, Muscle fibers; B, elastic fibers; C, parenchyma cells; D, starch; E, fragments of husk; F, aleurone grains; G, epidermal cells; H, stone cells; I, tube cells; J, bast fibers; K, fibro-vascular bundles; L, "skin" of seed; M, epidermis with gland cell (g) and stomata (s, s); N, palisade cells.

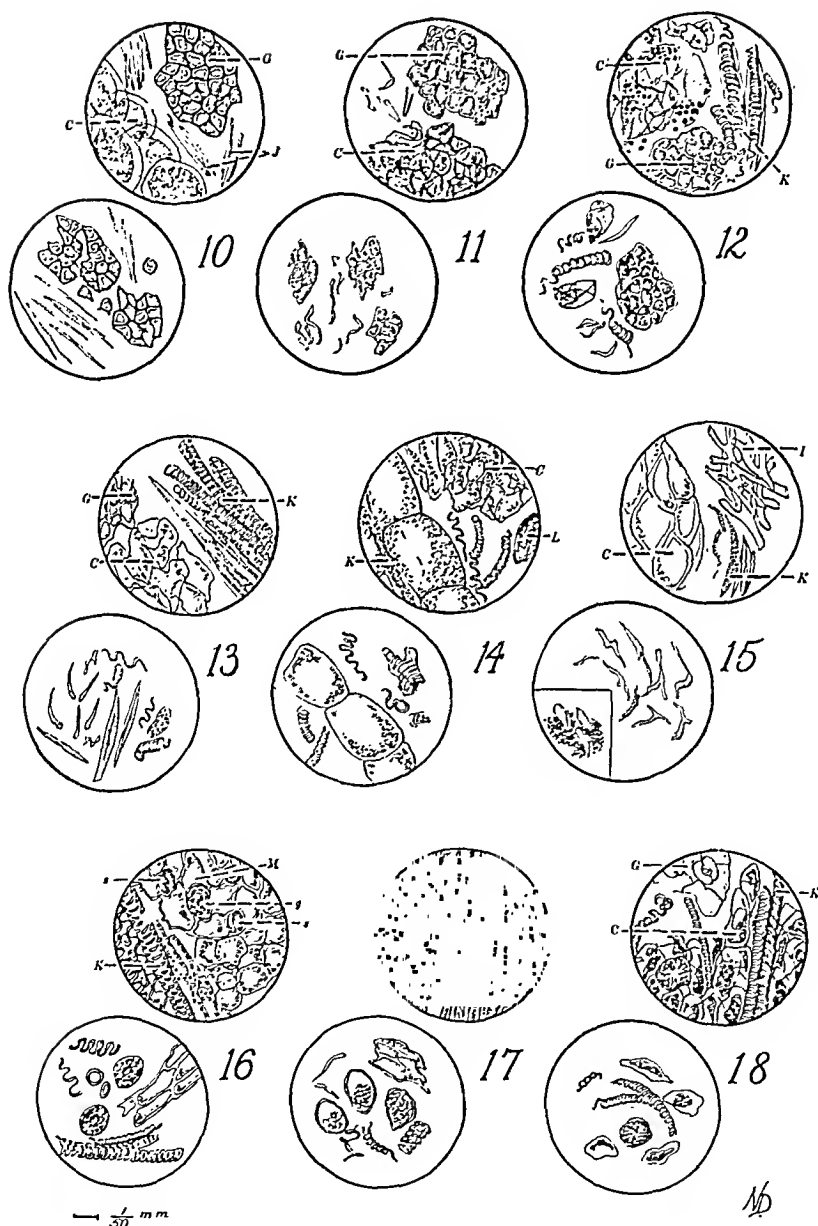


Plate II.—10, Apple; 11, raisin; 12, prune; 13, peach; 14, banana; 15, orange; 16, lettuce; 17, mustard greens; 18, spinach. (See Plate I legend for explanation of letters.)

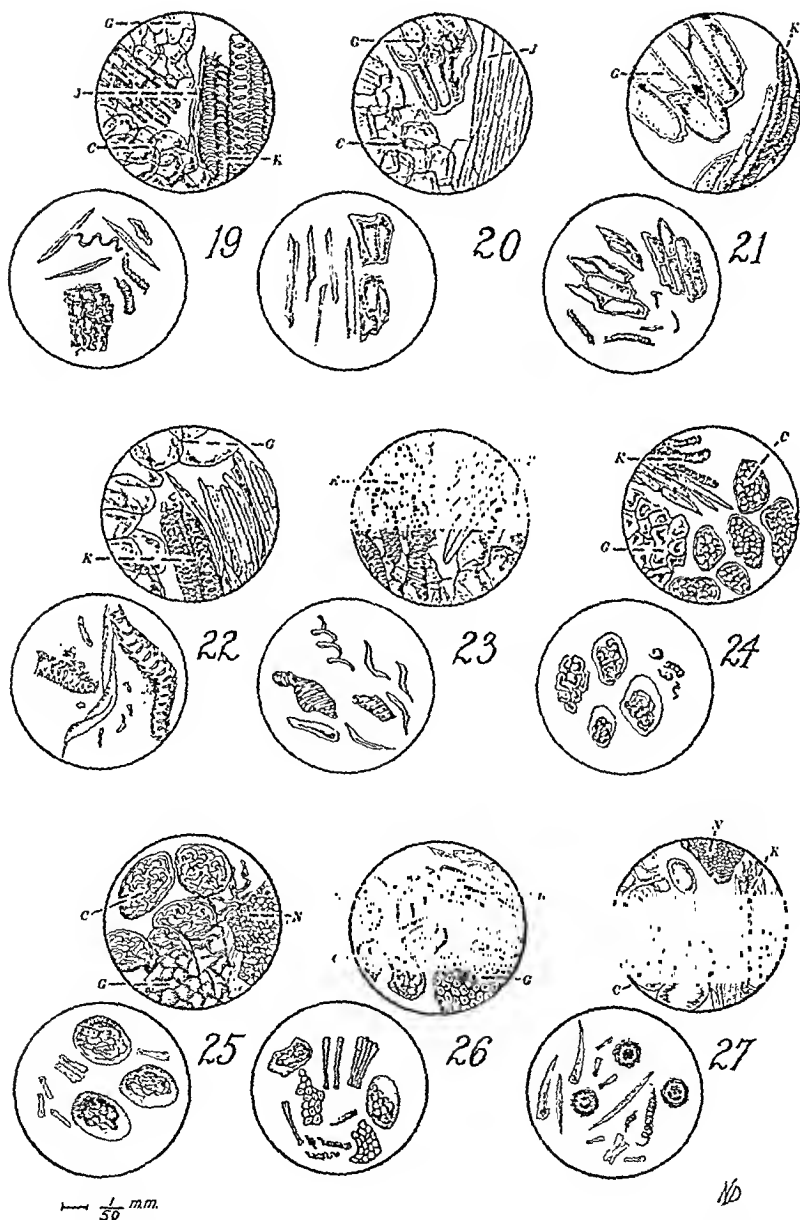


Plate III.—19, Cabbage; 20, celery; 21, onion; 22, beet; 23, carrot; 24, sweet potato; 25, pea; 26, lima bean; 27, string bean. (See Plate I legend for explanation of letters.)

The smaller particles from fibrovascular bundles are the characteristic lignated spirals and tubes, even fragments of which could be rarely ever confused with animal cells. Bast fibers, on the other hand, elongate lignated elements of the fibrovascular bundles, frequently simulate nematode larvae, the criterion of differentiation here again being the very thick walls and lack of internal structure in the plant cell. Stone cells, particularly of fruits, appeared frequently in feces; their shape and size at times closely resembled the eggs and cysts of parasites. Parenchyma cells were the most common of all cell types found singly, and, because of their shape and the presence of cytoplasm, might easily be confused with parasites. Because of the protection from digestion afforded by their cellulose walls, the remaining abundant cytoplasm allows the parenchyma cells of leafy vegetables, roots, and legumes to resemble helminth eggs, except for the lack of cellular organization or embryonic membranes. Parenchyma cells of bananas which frequently remain attached end-to-end have the appearance of minute tapeworm segments, but they lack internal organization or cuticular structures.

Starch granules observed in this study were for the most part destroyed by cooking; hence they were infrequently characteristic either in their staining reaction to iodine or in their structural appearance. Aleurone granules from grains often appeared much like minute protozoan cysts and might be very confusing unless closely studied.

Plates I, II, and III portray the characteristic appearance of the individual food elements studied both before and after digestion.

#### DISCUSSION

Foods ready to be served and the remnants occurring in feces have been studied from individuals whose digestive processes were apparently normal. Animal foods left no detritus that might cause any confusion in parasitologic diagnosis, but vegetables present a wide variety of remains, especially single-celled elements which closely simulate parasitic cysts or eggs. Fairly large, heavy-walled cells with shrunken cytoplasm, occurring as fecal remains and simulating helminth eggs, are usually derived from potatoes, leguminous seeds, such as peas and beans, or occasionally from various greens. Smaller cells and aleurone granules may simulate minute protozoan cysts.

It is not particularly difficult to determine into which general groups of plant foods fecal remnants may be classified, for as a rule some tissue fragments as well as single cells will occur, and a tentative diagnosis can be made from a consideration of the collective residues as to the foods eaten. Since such tissue fragments, because of their group organization, are not confusing, it is the undigested cells found in feces that are difficult to diagnose. However, their structure or their lack of structural organization should obviate errors in parasitic diagnosis, for, unlike protozoan cysts or helminth eggs, these cells are irregular in shape and size, their cytoplasm is shrunken, they rarely have nuclei, and they lack the internal order common in animals. Thus, even though a number of foods after apparently normal digestion have residues which might be confused with parasites, sufficient structures are left to prevent diagnostic mistakes.

## SUMMARY

1. An experimental study was conducted to ascertain which of the more common foods leave remnants in the feces which might be confused with intestinal parasites or their products.

2. A controlled nonresidue diet was used, to which various foods were added one at a time to allow study of the characteristic remnants of each food.

3. Foods which had no residue of any importance at all were animal products, such as milk, cheese, and eggs, and sea foods, such as oysters.

4. Animal foods which had residues were muscular fibers and liver, but their residues occurring in feces have no structures which could possibly be confused with parasites.

5. Remnants from plant foods included pieces of plant tissue, which should not lead to confusion, and single undigested cells, which might cause confusion. Of these, single parenchyma cells of leafy vegetables, roots, and legumes, epidermal gland cells, epidermal hairs, bast fibers, and aleurone granules are the most common elements likely to be mistaken for parasites.

6. Though many foods have confusing residues in the feces, there are sufficient differences in structure, including their inconstancy of size and shape, their irregular walls, their lack of nuclei and internal organization, to provide differential diagnosis.

I wish to acknowledge with gratitude the kindly council and guidance of Professor Ernest Carroll Faust.

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# THE PATHOLOGIC TISSUE CHANGES PRODUCED BY SULFATHIAZOLE AND SULFATHIAZOLINE IN RABBITS\*

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WITH THE TECHNICAL ASSISTANCE OF ANNA M. RULE AND LORAINÉ GROSCHIN

AS SHOWN by Rake, Van Dyke and Corwin,<sup>1</sup> the toxicity of sulfathiazole and sulfapyridine, including the pathologic tissue changes produced by oral administration, varies in mice, rats, and monkeys. Thus sulfathiazole was found more toxic for mice than sulfapyridine with the production of more pronounced tissue changes in the spleen and kidneys. In rats, however, sulfapyridine was found more toxic than sulfathiazole, with the production of more marked lesions in the kidneys. The most pronounced pathologic tissue changes produced by both compounds were observed in the kidneys due to the deposition of crystals in the collecting tubules with the production of uroliths resulting in obstruction associated in some instances with necrosis of the tubular epithelium. The spleen probably ranked next in histologic injury, largely confined to the germinal centers of the Malpighian corpuscles with hyperplastic changes sometimes associated with pyknosis of the surrounding lymphoblastic cells and their ultimate disappearance. The liver was less frequently involved, with the production of cloudy swelling sometimes associated with fatty infiltration and hyaline necrosis, while the lungs, brain, and meninges have uniformly shown no gross or microscopic evidences of injury.

In view of the importance of including gross and microscopic examinations of the organs in connection with studies bearing upon the toxicity of chemotherapeutic compounds, we have thought it advisable to determine the chemopathologic effects of sulfathiazole in rabbits by oral administration of the compounds; also of 2-sulfanilyl-3-5 dihydrothiazole (sulfathiazoline), a new thiazole compound synthesized by Raiziss and Clemence,<sup>2</sup> and reported by Raiziss, Severac, and Moetsch<sup>3</sup> as being of low toxicity for mice, rabbits, and dogs with therapeutic effects about the same as sulfathiazole in the treatment of experimental types II and III pneumococcal infections of mice and superior to sulfathiazole in the treatment of experimental staphylococcal infections of mice.

## EXPERIMENTAL

Normal adult rabbits were employed. In order to insure accurate dosage each compound was administered orally in capsules twice daily (10:00 A.M. and 3:00 P.M.) in doses of 0.050, 0.100, and 0.200 Gm. per kilogram of weight,

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for ten days in succession (20 doses), four animals being used for each dose. These amounts totaled 0.1, 0.2, and 0.4 Gm. per kilogram daily, equivalent to 7 to 28 Gm. per 70 kg. of weight daily over a period of ten days.

TABLE I

PATHOLOGIC TISSUE CHANGES PRODUCED IN RABBITS BY THE ORAL ADMINISTRATION OF SULFATHIAZOLE

| NO. | WEIGHT<br>(GM.) | DOSE PER<br>KILO*<br>(GM.) | RESULTS  | PATHOLOGIC TISSUE CHANGES |       |        |                                 |
|-----|-----------------|----------------------------|----------|---------------------------|-------|--------|---------------------------------|
|     |                 |                            |          | KIDNEYS                   | LIVER | SPLEEN | BRAIN,<br>CORD, AND<br>MENINGES |
| 1   | 1,950           | 0.050                      | Survival | -†                        | -†    | -†     | -†                              |
| 2   | 1,800           | 0.050                      | Survival | -                         | -     | -      | -                               |
| 3   | 1,800           | 0.050                      | Survival | -                         | -     | -      | -                               |
| 4   | 2,000           | 0.050                      | Survival | -                         | -     | -      | -                               |
| 5   | 2,000           | 0.100                      | Survival | +†                        | -     | +‡     | -                               |
| 6   | 2,100           | 0.100                      | Survival | -                         | -     | -      | -                               |
| 7   | 1,900           | 0.100                      | Survival | †                         | +§    | +      | -                               |
| 8   | 2,150           | 0.100                      | Survival | -                         | -     | +      | -                               |
| 9   | 2,100           | 0.200                      | Survival | +                         | -     | +      | -                               |
| 10  | 1,700           | 0.200                      | Survival | ++                        | ++    | ++     | -                               |
| 11  | 1,900           | 0.200                      | Survival | +                         | +     | +      | -                               |
| 12  | 2,150           | 0.200                      | Survival | +                         | -     | +      | -                               |

\*Orally in capsules twice daily for ten days (20 doses).

† = No gross or microscopic changes from the normal.

‡ = Dilatation of the convoluted tubules with crystals; ++ = dilatation of the convoluted and collecting tubules with some necrosis of the tubular epithelium and the formation of cellular casts; congestion with blood in Bowman's capsules.

§ = Slight cloudy swelling; ++ = cloudy swelling and some fatty infiltration associated with slight peripheral necrosis.

¶ = Slight hyperplastic changes in the germinal centers of the Malpighian corpuscles; ++ = same with pyknosis of the surrounding lymphoblastic cells.

TABLE II

PATHOLOGIC TISSUE CHANGES PRODUCED IN RABBITS BY THE ORAL ADMINISTRATION OF SULFATHIAZOLINE

| NO. | WEIGHT<br>(GM.) | DOSE PER<br>KILO*<br>(GM.) | RESULTS  | PATHOLOGIC TISSUE CHANGES |       |        |                                 |
|-----|-----------------|----------------------------|----------|---------------------------|-------|--------|---------------------------------|
|     |                 |                            |          | KIDNEYS                   | LIVER | SPLEEN | BRAIN,<br>CORD, AND<br>MENINGES |
| 1   | 1,800           | 0.050                      | Survival | -†                        | -†    | -†     | -†                              |
| 2   | 1,900           | 0.050                      | Survival | -                         | -     | -      | -                               |
| 3   | 2,000           | 0.050                      | Survival | -                         | -     | -      | -                               |
| 4   | 1,800           | 0.050                      | Survival | -                         | -     | -      | -                               |
| 5   | 1,700           | 0.100                      | Survival | -                         | -     | -      | -                               |
| 6   | 1,800           | 0.100                      | Survival | +†                        | +§    | -      | -                               |
| 7   | 1,600           | 0.100                      | Survival | +                         | -     | +‡     | -                               |
| 8   | 1,850           | 0.100                      | Survival | †                         | -     | +      | -                               |
| 9   | 2,100           | 0.200                      | Survival | ++                        | ++    | +      | -                               |
| 10  | 1,700           | 0.200                      | Survival | †                         | -     | -      | -                               |
| 11  | 1,700           | 0.200                      | Survival | ++                        | †     | ++     | -                               |
| 12  | 1,700           | "                          | "        | +                         | -     | -      | -                               |

\*Orally in capsules twice daily for ten days (20 doses).

† = No gross or microscopic changes from the normal.

‡ = Dilatation of the convoluted tubules with crystals; ++ = dilatation of the convoluted and collecting tubules with some necrosis of the tubular epithelium and the formation of cellular casts; congestion with blood in Bowman's capsules.

§ = Slight cloudy swelling; ++ = cloudy swelling and some fatty infiltration associated with slight peripheral necrosis.

¶ = Slight hyperplastic changes in the germinal centers of the Malpighian corpuscles; ++ = same with pyknosis of the surrounding lymphoblastic cells.

As shown in Tables I and II, all animals survived in the case of both compounds. There was no diarrhea or other clinical evidence of toxicity, except

some loss of weight in the case of the 8 animals receiving the largest doses of both compounds.

One week after the last dose all animals were sacrificed with macroscopic examination of the kidneys, liver, spleen, brain, spinal cord, and meninges. Sections were also prepared of these organs and subjected to microscopic examination.

### RESULTS

The results are summarized in Tables I and II. It will be observed that neither compound in dose of 0.05 Gm. per kilogram twice daily showed any gross or microscopic evidences of injury of the organs studied. Both, however, have shown evidences of injury of the kidneys, liver, and spleen in dose of 0.1 Gm. per kilogram twice daily in the case of some animals which were more frequent, and sometimes more pronounced in those given 0.2 Gm. per kilogram twice daily. No gross or microscopic evidences of injury were found in the meninges, brain, and spinal cords of any of the animals, including those receiving the largest amounts of both compounds.

The usual changes in the kidneys consisted of slight enlargement with hyperemia due to dilatation of the convoluted or collecting tubules sometimes associated with tubular necrosis, cellular casts, and occasionally blood in the capsules of Bowman. The latter changes were found only in rabbits receiving the largest doses of both compounds.

The spleen was next in the frequency of tissue injury, and especially in the case of those animals receiving the largest amounts of both compounds. When involved, it was slightly enlarged and hyperemic, usually showing upon microscopic examination some hyperplasia of the germinal centers of the Malpighian corpuseles occasionally associated with congestion and with pyknosis of the surrounding lymphoblastic cells.

Both compounds in dose of 0.1 Gm. per kilogram twice daily showed only occasionally a slight cloudy swelling of the lobules of the liver. In the case of animals given 0.2 Gm. per kilogram twice daily, however, the degree of cloudy swelling was more pronounced, sometimes associated with fatty infiltration and slight necrosis of the peripheral cells of the lobules. There were no evidences, however, of injury or obstruction of the biliary ducts.

Like sulfapyridine, it would appear, therefore, that the main injury from sulfathiazole and sulfathiazoline is to be found in the kidneys, mainly due to obstruction of the tubules by crystals of the acetylated compounds. This may result in hydronephrosis, with enlargement of the organs which may progress in some instances to tubular necrosis.

Insofar as comparative results of the two compounds are concerned, it would appear that both are of extremely low toxicity for rabbits by oral administration and of about equal toxicity insofar as injury of the kidneys, liver, and spleen are concerned. As previously stated, neither compound showed any clinical, macroscopic, or microscopic evidences of injury of the central nervous system.

### SUMMARY

1. Both sulfathiazole and sulfathiazoline are of low toxicity for rabbits by oral administration. Doses of 0.05, 0.1, and 0.2 Gm. per kilogram twice daily for ten days in succession (20 doses) were well borne with no fatalities.

2. Neither compound showed any macroscopic or microscopic evidences of injury of the meninges, brain, or spinal cord.

3. In dose of 0.05 Gm. per kilogram twice daily for 20 doses neither compound showed any gross or microscopic injuries of the kidneys, liver, or spleen.

4. Both compounds in dose of 0.1 and 0.2 Gm. per kilogram twice daily for 20 doses showed injury of the kidneys largely due to obstruction of the tubules by crystals of acetylated compounds, sometimes associated with tubular necrosis. These were the most frequent lesions observed. Both compounds were about the same in their production.

5. Both compounds in dose of 0.1 and 0.2 Gm. per kilogram twice daily for 20 doses were about equal in the production of injury of the liver, usually expressed as cloudy swelling sometimes associated with slight fatty degeneration and necrosis of the peripheral cells of the lobules.

6. Both compounds in dose of 0.1 and 0.2 Gm. per kilogram twice daily for 20 doses were about equal in the production of injury of the spleen, usually expressed by slightly hyperplastic changes in the germinal centers of the Malpighian corpuscles.

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## SULFANILAMIDE FOR THE TREATMENT OF GONORRHEA OF THE ANAL CANAL

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WITH THE TECHNICAL ASSISTANCE OF MIRIAM GREEN

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IN A previous report<sup>1</sup> I emphasized that sulfanilamide administered orally is effective in the treatment of anal gonorrhea in the absence of localized suppurations or poorly draining sinuses. The plan of treatment consisted of the administration of 1 Gm. of sulfanilamide every four hours for 24 to 30 doses, which resulted in a concentration of from 6 to 11 mg. of free sulfanilamide in each 100 c.c. of blood. Thereafter, the drug was reduced to from 3 to 4 Gm. daily and was continued for a period of from four to five weeks. Berck,<sup>2</sup> using smaller amounts of sulfanilamide, has also noted good results in the treatment of this lesion.

The following case histories are illustrative of the effectiveness of sulfanilamide\* therapy for gonorrhea of the anal canal.

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\*Sulfathiazole or sulfadiazine is now preferred.

CASE 1.—A 34-year-old white woman was seen on March 22, 1938, with intermittent discomfort of the anus and occasional sharp pain on defecation. About three years before, she had received intensive treatment for genital gonorrhea. Proctologic examination now revealed a superficial fissure with a deep, reddened, infected, and edematous crypt of Morgagni on the posterior arc of the anal canal. Material obtained from the interior of the involved crypt with the aid of a bent platinum wire loop (which was inserted through small anal speculum) revealed numerous leucocytes and gram-negative intracellular diplococci resembling gonococci. The sedimentation time was above normal. Six grams of sulfanilamide were given daily by mouth for five days; 4 Gm. daily for the succeeding eleven days, and thereafter 3.3 Gm. daily for a period of over five weeks. Instrumental examination was avoided for a period of seventeen days. At the end of that time, as well as during subsequent re-examinations, no gram-negative intracellular diplococci were observed. The patient remained cured when last seen on May 12, 1939.

CASE 2.—A 30-year-old white woman had received an undetermined amount of sulfanilamide by mouth (the exact amount of the drug taken could not be ascertained) and azosulfamide solution parenterally in June, 1937, for the treatment of genital gonorrhea. On Dec. 16, 1938, she had complained of sharp shooting pains in the muscles of the thighs and in various joints. The physical examination was normal, except for reddening, injection, and tenderness of the anal canal and lower rectum. Thin mucopurulent material obtained from an anteriorly situated inflamed crypt of Morgagni showed gram-negative intracellular diplococci. On Sept. 29, 1939, tenderness in the region of the right Bartholin gland was observed, which was relieved by external heat. On Jan. 15, 1940, the patient again complained of pain in the muscles of the left thigh; this persisted intermittently in spite of conservative therapy (hot hip baths, salicylates). Reexamination on May 1, 1940, showed injection of the anal canal. A Gram stain of the material obtained from the interior of an anteriorly situated crypt of Morgagni again revealed gram-negative intracellular diplococci and many leucocytes. Sulfanilamide therapy was begun on May 25, giving 4.5 Gm. daily for the first four days, and 2 to 4 Gm. daily thereafter until a total of 50 Gm. of sulfanilamide had been taken. Re-examination of the anal canal on June 14, and again on July 22, showed a few leucocytes per field; no gram-negative intracellular diplococci were found. The patient has remained symptom-free to date.

#### COMMENT

These results show that sulfanilamide appears to be effective in the treatment of uncomplicated gonorrhea of the anal canal. The plan of sulfanilamide therapy employed has been found safe for the patient who is under daily medical supervision.

Gonorrheal proctitis is "a disease one sees only if one looks for it" (Jullien-Martin). It is realized that gonococci may remain alive and virulent for years in the anal structures,<sup>3</sup> and that reinfection of the genital tract may occur from the anal focus.<sup>4-7</sup> Except for cases of pederasty, contamination in girls and women is believed to take place during the act of defecation, when a simultaneous expulsion of gonococci-laden discharge from the vagina and a normal eversion of the anal mucous membrane occur. Suitable biologic conditions for the inception and maintenance of gonorrheal infection are furnished by the transitional epithelium of the intermediate zone of the anal canal,<sup>6</sup> the anal ducts, and the crypts of Morgagni.<sup>8</sup> The infection may spread to the deeper perianal and perirectal structures through the anal ducts which lead to racemose multiglandular structures located in the perianal tissues, and penetrate the sphincter muscles, especially the internal sphincter,<sup>9-11</sup> producing suppurations and subsequent fistulas.

## SUMMARY

Sulfanilamide has been successfully employed in the treatment of uncomplicated gonorrhea of the anal canal.

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876 PARK AVENUE

## BACTERIUM COLI ANAEROGENES SEPTICEMIA\*

### REPORT OF A CASE WITH RECOVERY

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THE incidence of infection with *Bacterium coli anaerogenes* is very small, and only a few cases of septicemia produced by this organism have been reported. Lembke<sup>1</sup> in 1896 originally described this organism which he recovered from dog dejecta and named it *B. coli anaerogenes*. In 1900 Migula,<sup>2</sup> in his *System of Bacteriology*, called it *Bacterium lembkei*. Apparently, the same organism was reported by Castellani<sup>3</sup> in 1907 as *Bacillus ceylonensis*; he recovered it in four cases of a febrile disease in Ceylon. In two of these cases he obtained the organism by blood culture. Wilson<sup>4</sup> the next year isolated such an organism in six cases of cystitis. Houston<sup>5</sup> in 1906, and Swan<sup>6</sup> in 1909, found similar bacteria in the stools of healthy persons. In 1906 Dudgeon<sup>7</sup> isolated a pathogenic bacillus of this type from the secretion of an enlarged prostate gland. Also in this year similar organisms were found by Catheart<sup>8</sup> among the flora of "blown" tins of preserved food. Nabarro<sup>9</sup> in 1923 isolated from cases of epidemic and other forms of diarrhea, which occurred mainly in children, a group of bacteria which he called *B. coli anaerogenes*. However, some of these strains were later shown by Kerrin<sup>10</sup> in 1928

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to be identical culturally and serologically with the *B. dysenteriae* Sonne. Bamforth<sup>11</sup> in 1923 concluded in his studies that it was serologically related to some other members of the coli-dysentery group, but not to the Sonne organism. In 1939 Savino and co-workers<sup>12</sup> demonstrated the presence of bacteria of the *Coli anaerogenes* group in gray rats caught in Buenos Aires.

The organism under discussion is known by several other names:<sup>13-14</sup> *Bacterium anaerogenes chester*, *Bacillus belfastiensis*, *Castellanus coli anaerogenes*, *Escherichia anaerogenes*, and *Eberthella belfastiensis*. *Bacterium coli anaerogenes*, according to Bergey,<sup>13</sup> is a small, nonmotile, gram-negative rod. On agar the growth is slow, producing circular, grayish-white, smooth colonies on plates, and a grayish-white streak on the slant. There is no liquefaction in the gelatin stab; litmus milk is turned acid and coagulates; broth is made turbid; a brown growth is produced on potato, and indol is formed. Acid, but no gas, is observed in dextrose, levulose, arabinose, lactose, maltose, sucrase, raffinose, mannitol, sorbitol, and isosorbitol; no acid from inulin, salicin, dulcitol, glycerol, or adonitol. It is a facultative anaerobic, growing best at 37° C., and is found in the intestinal tract of man where it may produce symptoms that resemble those of typhoid fever. Ford<sup>14</sup> stated that *B. coli anaerogenes* is virulent to guinea pigs: one cubic centimeter of a twenty-four-hour broth culture will produce enteritis, peritonitis, congestion of the liver and lungs, and cloudy swelling of the kidneys.

#### REPORT OF CASE

A white woman, aged 47 years, was admitted to Stuart Circle Hospital with menstrual bleeding of three months' duration. Pelvic examination revealed a large tumor, which filled the right side of the pelvis. A diagnosis of multiple fibroids of the uterus was substantiated at operation two days following admission, but there was also found a large intraligamentous ovarian cyst of the multilocular type which filled the pelvis. The left ovary appeared normal. Both tubes were in a state of hydrosalpinx. The surgery undertaken consisted of a right oophorectomy, supravaginal hysterectomy, bilateral salpingectomy and a prophylactic appendectomy; the abdomen was closed without drainage. The patient left the operating room in good condition.

The postoperative course was marked by considerable nausea and vomiting for which the patient received parenterally solutions of dextrose and normal saline. Abdominal distention was relieved by a continuous suction apparatus attached to an inlying duodenal tube and by the low gravity method of colonic irrigation. During the first postoperative day (beginning twenty-four hours after operation) the temperature rose to 100° F. and on the second day it became suddenly elevated to 105.4° F. A severe chill was experienced, and the pulse rate reached 120 per minute. That night, and the following day, the temperature gradually came down to 100° F. On this, the third postoperative day, a transfusion of 500 c.c. of blood was given by the direct method. Blood was taken for a culture, and this was repeated the following morning. From both of these there was recovered a small, nonmotile, gram-negative rod, which could not be readily identified. During the next two days the patient had repeated chills at which times the temperature went as high as 104° F.

Sulfanilamide, 15 grains every four hours, was started on the fifth postoperative day. The night of the sixth day the patient was suddenly stricken with an attack of dyspnea, chills, and a temperature of 105° F. She appeared cyanotic; the heart action was accelerated, and the blood pressure increased from systolic 150 to 200 mm. Hg in less than an hour. The heart and lungs were found to be clear to auscultation and percussion. Since a pulmonary embolus was suspected, the patient was placed in an oxygen tent. Her dyspnea was benefited and her color returned to normal under oxygen therapy and was maintained for two

days. However, the chills and fever (maximum 105° F.) continued despite all therapy. A pelvis pyelophlebitis was next considered as a possible cause of these symptoms. Sulfathiazole was then given orally instead of sulfanilamide, but this was also discontinued in thirty-six hours, after 120 grains were ingested without any demonstrable relief. Although some pneumonitis was seriously suspected, no definite lung findings could be detected, except for a few hypostatic râles in both bases. A roentgenogram of the chest was not taken. On the eighth day a second transfusion of 500 c.c. of whole blood was given without any apparent improvement in the patient's condition. The next day a widespread herpes labialis became evident, and this caused the patient much discomfort. The chills and fever (maximum 103.5° F.) continued with varying intensity until the thirteenth postoperative day. On the fourteenth day the stitches were removed, revealing a well-healed wound free of infection. The patient improved gradually under symptomatic treatment and was discharged as cured on the thirty-second day following her operation.

Throughout the period of infection the leucocyte count remained relatively low, between 8,000 and 10,000 cells per cubic millimeter, and reached 14,700 cells per cubic millimeter only once—on the tenth day. The hemoglobin on admission was 67 per cent (Newcomer), and during the postoperative period it dropped to 45 per cent; it finally reached 58 per cent before the patient left the hospital. The sedimentation index on the ninth postoperative day was 24 mm. in sixty minutes. The urine contained albumin in varying amounts, from a faint trace to four plus on the eighth postoperative day; it finally became negative on the fifteenth day. Five days following the patient's discharge, a report was received from the Virginia State Board of Health, which stated that the organism isolated from the blood culture was classified as belonging to the para-colon group, culturally resembling *Bacterium coli anaerogenes* in that it failed to produce gas in certain carbohydrates. A similar report was received through the Virginia State Board of Health from the New York State Board of Health and was verified by one of us (F. W. S.). Two months following her discharge, the patient returned to the hospital for a physical examination, at which time blood for agglutinations was taken. A positive titer of 1:1,500 dilution against *Bacterium coli anaerogenes* was discovered.

#### COMMENT

The focus of infection in this case was not determined. Since the *Bacterium coli anaerogenes* is an inhabitant of the intestinal tract in some people, it would seem logical to assume that the primary focus of infection was in the lymphatic vessels leading from the intestinal canal. From here, because of lowered resistance following the operation and the initial anemia which was still further lowered owing to the loss of blood during the operation, the bacteria might readily have spread through the lymphatic channels into the blood stream where a secondary focus could have been set up in the congested pelvic veins resulting in pyelophlebitis, and finally a septicemia. The patient was so overwhelmingly toxic throughout her febrile course that she was not expected to live on several occasions. Neither the blood transfusions nor the sulfonamide preparations (sulfanilamide, 90 grains, and sulfathiazole, 120 grains) seemed to have any therapeutic effect in shortening the course of the infection.

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## THE ANTAGONISM BETWEEN ANESTHETIC STEROID HORMONES AND PENTAMETHYLENETETRAZOL (METRAZOL)\*

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IT IS well known that analeptic, convulsive drugs, such as picrotoxin or pentamethylenetetrazol (metrazol), counteract the narcotic effect of certain anesthetics, especially the barbiturates and tribromethanol (avertin). Since the relevant literature has recently been reviewed in detail by Beecher,<sup>1</sup> it will not be necessary to discuss it here. It has also been found that some steroid compounds, especially those endowed with hormone action, act as anesthetics if given intraperitoneally or intravenously, that is to say, if administered in a manner which permits the rapid establishment of a high steroid concentration in the blood.<sup>2-7</sup> This type of anesthesia produced by naturally occurring hormonal substances proved very satisfactory for use in laboratory animals, since the safety margin between the anesthetic and lethal dose is comparatively great, and the anesthesia is accompanied by very pronounced muscular relaxation. Steroid anesthesia is especially suitable when long-lasting narcosis is required, but it cannot be used satisfactorily in cases in which a deep anesthesia of short duration is needed because a dose sufficient to produce deep anesthesia, at any one time in the course of its action, usually maintains the state of narcosis for several hours. It appeared of interest, therefore, to establish whether pentamethylenetetrazol could arouse animals from the hormone anesthesia whenever desired.

### EXPERIMENTAL

In our first experiment young male albino rats were divided into three equal groups. In the first two groups deep anesthesia was produced by the intraperitoneal administration of desoxyeortieosterone acetate (D.C.A.) in peanut oil solution. Thirty minutes after the injection of this hormone, when all animals were in complete narcosis, one of the groups pretreated with desoxyeortieo-

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sterone acetate and a group of controls not pretreated received a subcutaneous injection of pentamethylenetetrazol in aqueous solution. Table I summarizes the results of this experiment.

TABLE I

## ANTAGONISM BETWEEN DESOXYCORTICOSTERONE ACETATE AND PENTAMETHYLENETETRAZOL

| TREATMENT   | NO. OF ANIMALS | AVERAGE BODY WEIGHT IN GM. | AVERAGE DEGREE OF ANESTHESIA | CONVULSIONS | DEATHS |
|---|----------------|----------------------------|------------------------------|-------------|--------|
| Desoxycorticosterone acetate 4 mg. per 0.2 ml.  | 7              | 35                         | +++                          | 0           | 0      |
| Pentamethylenetetrazol 5 mg. per 0.2 ml. and Desoxycorticosterone acetate 4 mg. per 0.2 ml. | 7              | 36                         | Trace                        | 0           | 1      |
| Pentamethylenetetrazol 5 mg. per 0.2 ml.  | 7              | 36                         | 0                            | +++         | 7      |

Our data clearly indicate that pentamethylenetetrazol antagonizes the anesthetic action of desoxycorticosterone acetate. It should be stated that this is not merely a prevention but an actual interruption of the anesthesia, since the animals in the group in which desoxycorticosterone acetate was administered before the pentamethylenetetrazol, were deeply anesthetized at the time the latter drug was administered, yet five minutes later they were on their feet again and showed only slight traces of a narcosis, such as uncertainty of their movements and unwillingness to run about. It is even more interesting to note that in the condition of hormone anesthesia, otherwise lethal doses of pentamethylenetetrazol did not kill a single animal and indeed prevented even the appearance of convulsions. The animals not pretreated, which died from pentamethylenetetrazol overdosage, developed very pronounced lung edema. This was also prevented by desoxycorticosterone acetate. Thus we can see that the antagonism between desoxycorticosterone acetate and pentamethylenetetrazol is mutual.

TABLE II

## ANTAGONISM BETWEEN PROGESTERONE AND PENTAMETHYLENETETRAZOL

| TREATMENT   | NO. OF ANIMALS | AVERAGE BODY WEIGHT IN GM. | AVERAGE DEGREE OF ANESTHESIA | CONVULSIONS | DEATHS |
|---|----------------|----------------------------|------------------------------|-------------|--------|
| Progesterone 5 mg. per 0.2 ml.  | 10             | 45                         | +++                          | 0           | 0      |
| Pentamethylenetetrazol 3 mg. per 0.2 ml. twice and progesterone 5 mg. per 0.2 ml. | 12             | 47                         | 0                            | 0           | 0      |
| Pentamethylenetetrazol 3 mg. per 0.2 ml. twice                                    | 10             | 46                         | 0                            | +++         | 10     |

A second series of experiments was performed under very similar conditions in order to establish whether progesterone, which is also a potent anesthetic when given by the intraperitoneal route, would likewise prove to be an antagonist of pentamethylenetetrazol. The results of this experiment are summarized in Table II.

In this experiment pentamethylenetetrazol was administered in two injections. The first dose of 3 mg. aroused the animals from progesterone anesthesia

for a period of about fifteen minutes, after which they began to relapse into narcosis. Permanent awakening was produced, however, by a second dose of 3 mg. The controls not pretreated showed marked convulsions even under the influence of the first injection of pentamethylenetetrazol, and one animal died from lung edema. The second injection produced fatal lung edema in all the remaining controls.

#### DISCUSSION

Our experiments indicate that pentamethylenetetrazol can interrupt steroid anesthesia so that in this respect the narcosis produced by progesterone and desoxycorticosterone acetate resembles that elicited by barbiturates or tribromethanol. It is also evident that the convulsions and the fatal lung edema usually produced by lethal doses of pentamethylenetetrazol are very effectively prevented by steroid anesthetics. In this respect the steroids again resemble the barbiturates which, according to Maloney<sup>8</sup> likewise raise resistance to convulsive drugs. The anesthetic steroids and pentamethylenetetrazol may be regarded as potent mutual antagonists of each other's pharmacologic actions.

#### SUMMARY

Experiments on young albino rats indicate that the anesthesia produced by the intraperitoneal administration of steroid hormones (progesterone or desoxycorticosterone acetate) can be interrupted at will by pentamethylenetetrazol (metrazol). Conversely, fatal doses of pentamethylenetetrazol are readily tolerated without causing either convulsions or lung edema by rats receiving suitable doses of anesthetic steroid hormones.

I am indebted to Doctors Gregory Stragnell and Erwin Schwenk, of the Schering Corporation, New Jersey, for the progesterone and desoxycorticosterone acetate, and to Dr. Fred B. Western, of the Bilhuber-Knoll Corporation, Orange, N. J., for the metrazol used in these experiments.

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# CLINICAL CHEMISTRY

## PLASMA PROTEINS IN THERAPEUTIC FEVER\*

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CHANGES in the plasma proteins have been observed in various infections and have consisted in the main of a reduction in the albumin and increases in the globulin and fibrinogen.<sup>1-5</sup> A study of the factors responsible for the edema occasionally resulting during the course of therapeutic malaria has revealed also rather marked changes in the plasma protein fractions.<sup>6</sup>

The present communication is concerned with the changes in the plasma proteins of nine patients to whom malaria, artificial fever, and typhoid vaccine fever were administered for treatment of syphilis of the central nervous system.

*Material.*—Nine male patients, ranging from 40 to 51 years of age, were hospitalized for fever therapy of general paresis. Physical and laboratory procedures revealed no other complicating diseases.

*Methods.*—Six patients were given tertian malaria only; one patient, artificial fever and malaria; one patient, tertian and quartan malaria and typhoid vaccine fever; and one patient, typhoid vaccine fever.

Malarial therapy was administered by the intravenous injection of 3 to 4 c.c. of malarial blood. The incubation period ranged from one to seven days. True tertian or quartan fever was not encountered, the fever being of the mixed or quotidian type. Malaria was terminated in seven patients after 10 to 13 paroxysms by the daily administration of 30 grains of quinine sulfate for one week. One patient experienced only four paroxysms of fever above 104° R. after two inoculations with tertian malaria and one with quartan malaria.

Typhoid vaccine fever was induced by the intravenous injection of increasing doses of mixed typhoid vaccine (*Bacillus typhosus*, paratyphoid A and B), administered usually three times a week. One patient was given eleven bouts of fever, and a second, sixteen bouts of fever.

Artificial fever was induced by means of the inductotherm and humidified air cabinet. Body temperature was raised to level of 105° to 106° R., and prolonged for two hours at each treatment. A total of fourteen treatments was given to one patient over a thirty-eight-day period.

The daily hospital diet consisted roughly of the following: protein 80 Gm., carbohydrate 315 Gm., and fats 85 Gm. When malarial fever was present and food intake diminished, the daily diet was reinforced by the addition of at least one quart of milk and two eggs (protein 42 Gm., carbohydrate 50 Gm., and fat 52 Gm.). After termination of the malarial fever the patient was given a high vitamin and high calorie diet and 20 grains of ferrous sulfate daily.

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Sodium chloride was administered to seven patients during fever therapy as a result of the observations of Judd,<sup>7</sup> who felt that patients were better able to tolerate malarial therapy when 10 grains of this salt were given on those days when body temperature rose above 103° R. Larger doses, ranging from 3 to 6 Gm. daily, were given to our patients. The sodium chloride was omitted when therapeutic fever was terminated with the exception of one patient, who continued to receive 6 Gm. daily for one week.

Studies were made of the plasma proteins, hematocrit, red blood cell, white blood cell, and hemoglobin (Sahli) levels on venous blood samples in the resting state. The total protein was obtained by the determination of the total nitrogen by a modified micro-Kjeldahl.<sup>8</sup> The nonprotein nitrogen was determined by the method of Folin and Wu,<sup>9</sup> and the difference between the total nitrogen and the nonprotein nitrogen was multiplied by the factor 6.25 to give the total protein. The albumin and globulin were determined by the sodium sulfate method of Howe.<sup>10</sup> The fibrinogen was determined by precipitation as fibrin by the method of Cullen and Van Slyke,<sup>11</sup> and the nitrogen was determined by the difference between the total nitrogen and the nitrogen in the fibrinogen-free filtrate. In the last series of studies the fibrinogen was precipitated as above, and the nitrogen content of the fibrin was determined directly. Potassium oxalate in the concentration of 2 mg. per cubic centimeter was used as the anticoagulant.

## RESULTS

*Malaria.*—Intravenous malarial inoculation was followed in seven of eight patients by a slight or a marked and rapid fall in albumin during the incubation period and before any fever occurred. One patient experienced no fever for thirteen days following his first inoculation with tertian malaria. Albumin fell from 4.82 to 4.01 Gm. during this period. A second patient showed a reduction in albumin from 5.1 to 4.3 Gm. three days after inoculation with tertian malaria and one day before any fever resulted. Only one patient showed an increase in albumin, from 4.37 to 4.70 Gm. Globulin and fibrinogen showed fluctuating changes during the incubation period; the changes in globulin ranging from a reduction of 25 per cent to an increase of 45 per cent, and those of fibrinogen from a fall of 50 per cent to an increase of 300 per cent.

The onset and recurrence of the malarial fever were accompanied by a rapid, progressive, and marked reduction in albumin (Chart 1). The reductions in albumin ranged from 30 to 40 per cent, falling from prefebrile values of 3.67 to 5.1 Gm. (average 4.53 Gm.) to levels of 2.23 to 3.25 Gm. (average 2.89 Gm.). Globulin fluctuated considerably during malarial fever but showed no consistent trend. Increases of 11 to 65 per cent occurred in seven patients. In the eighth patient globulin values throughout malaria remained below the original level of 3.03 Gm., with a maximum reduction of about 32 per cent. Fibrinogen also showed no consistent trend during active malaria. Of the eight patients, one showed significant reductions as much as 50 per cent below prefebrile values; two, an increase and then a progressive fall below initial levels; two, fluctuations above and below original levels; and three, similar values or an increase.

The fall in albumin ceased immediately or within a few days after the administration of quinine and the cessation of malarial fever (Chart 1). Albumin values then rose rapidly, reaching prefebrile levels in six patients in fourteen to twenty-four days. In one patient the prefebrile value was reached only after seventy days. The malarial fever of the eighth patient ceased spontaneously, and no quinine was administered. Sixteen days after the last febrile reaction albumin differed only little from the lowest level obtained during

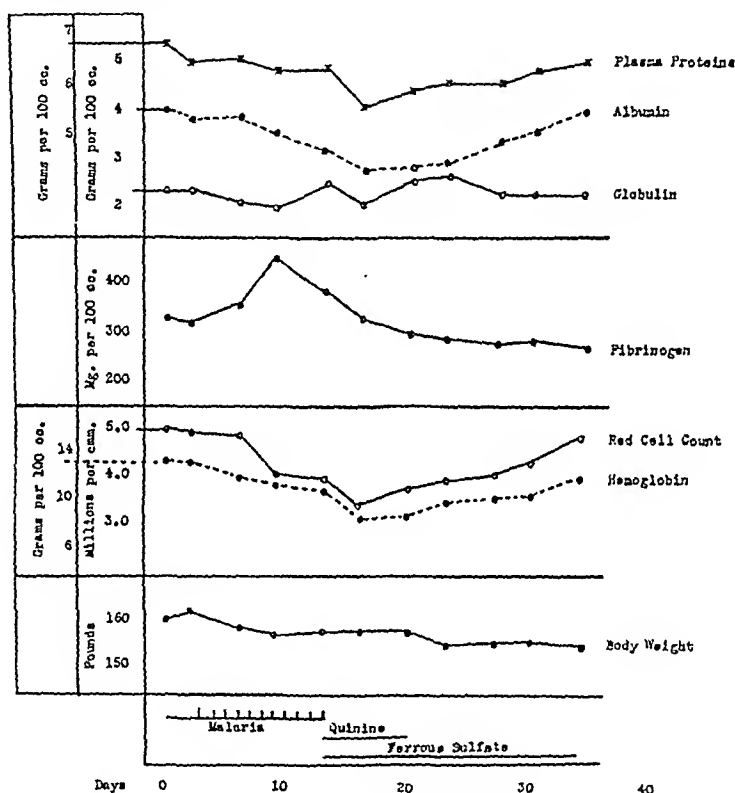


Chart 1.—Plasma proteins in therapeutic malaria.

malarial fever, namely, 3.6 and 3.23 Gm. Globulin remained increased for only a short period after malarial fever was terminated, and then fell slowly to reach prefebrile values in ten to eighteen days. The fibrinogen values returned to original levels fairly rapidly, in about seven days. Fibrinogen and globulin values of the patient who received no quinine therapy remained elevated even after two weeks' time.

*Typhoid Vaccine Fever.*—Only slight fluctuations in albumin, ranging from a fall of 9 per cent to an increase of 13 per cent, occurred in two patients to whom 11 and 16 bouts of typhoid vaccine fever were administered over periods of twenty-four and thirty-nine days, respectively (Chart 2). This is in marked contrast to the severe reductions of 30 to 40 per cent which were found during malaria. Globulin also showed fluctuations in both directions but of a smaller magnitude than in malaria. Both albumin and globulin values when treatment was terminated approximated those present before therapy. Fibrinogen in-

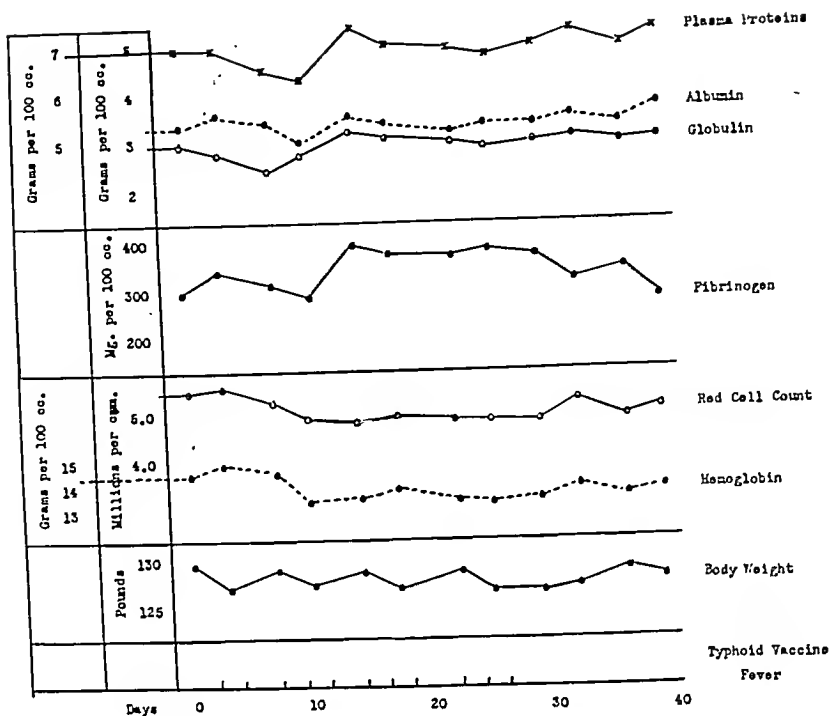


Chart 2.—Plasma proteins in fever induced by typhoid vaccine.

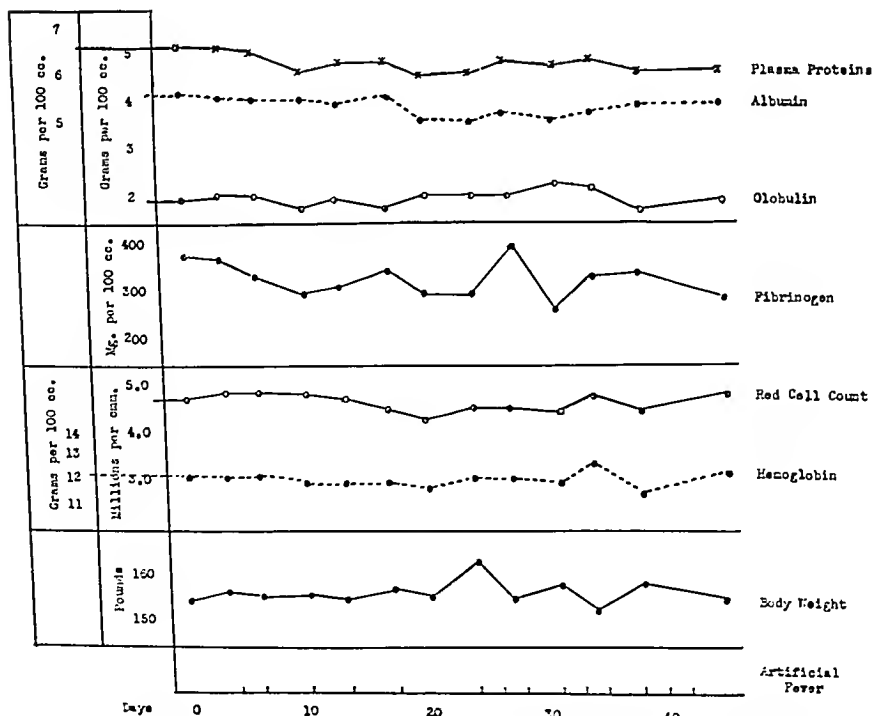


Chart 3.—Plasma proteins in fever induced by artificial means (inductotherm).

creased in both patients as a result of typhoid vaccine fever, in one 33 per cent and in the second 40 per cent. In the former fibrinogen returned to its original level within seven days after treatment ceased, but in the latter fibrinogen remained increased, more than 200 per cent, as late as thirty-one days thereafter.

*Artificial Fever.*—Repeated administration of artificial fever resulted in only a slight reduction of the albumin, the maximum fall being 13 per cent below the prefebrile level (Chart 3). The final value, however, after a course of 14 treatments, over a thirty-eight-day period, differed only little from that before treatment. The changes in globulin fluctuated equally, about 12 per cent, in both directions. Fibrinogen values during therapy tended to fall as much as 30 per cent below prefebrile levels but remained within normal limits. Three weeks after therapy was terminated the patient was inoculated with tertian malaria and experienced a 30 per cent reduction in albumin as a result of malarial fever (Chart 1).

#### DISCUSSION

The changes in the plasma protein fractions as a result of therapeutic malaria consist of a marked reduction in albumin, fluctuating changes in the globulin, and generally increased fibrinogen. Similar changes have been found in hookworm disease accompanied by severe anemia,<sup>12</sup> less marked changes in tuberculosis,<sup>13</sup> and other infections.<sup>4</sup> Lloyd and Paul,<sup>1</sup> by refractometric methods, found marked reductions in albumin and slight falls in the total globulin in patients with malignant and benign tertian malaria. The degree of albumin reduction seemed to parallel the intensity of the infection, greater reductions occurring in patients with high temperatures than in those with subfebrile infections. Similar changes were found by these authors in patients with typhoid fever. The rise in albumin to normal was said to be more rapid following malaria than typhoid fever, and was attributed to the presence of a specific drug, quinine, for the treatment of the former. It is possible that the absence of quinine therapy in one of our patients may be responsible for the only slight rise of albumin from its lowest level of 3.23 Gm. during malaria to 3.6 Gm. sixteen days after fever spontaneously ceased, and for its failure to rise to the original level of 4.82 Gm. during this period. Lloyd and Paul<sup>1</sup> observed several untreated patients with malaria in whom parasites were present in the peripheral blood stream but no fever was present. All showed the characteristic protein changes, a reduction in albumin, and variable changes in globulin.

The changes in the serum protein fractions as a result of malaria were much more pronounced than those found in fever produced either by mixed typhoid vaccine or by artificial means. This may be due to (a) the rapid recurrence and greater amount of fever; (b) the presence of blood destruction; and (c) the presence of an infectious process in malaria. Active malarial fever required from ten to seventeen days in contrast to thirty-eight days for artificial fever, and twenty-four and thirty-nine days for typhoid vaccine fever. The paroxysms of malaria were usually of daily occurrence, whereas treatment in the other types of fever was given two or three times each week. The total amount of fever above 100° R. was also greater in malaria, an average of one



hundred twelve hours, in contrast to eighty-seven hours and fifty-five hours for typhoid vaccine fever and artificial fever, respectively. On the other hand, the plasma protein changes of the eight patients given malarial therapy differed only little, regardless of the number and frequency of the paroxysms, the maximum temperature experienced, the total amount of fever above  $100^{\circ}$  R., or the total duration of malarial therapy. The number of malarial paroxysms (temperature  $103^{\circ}$  R., and above) ranged from four to thirteen, the total amount of fever above  $100^{\circ}$  R. from seventy-one to one hundred fifty-four hours, the duration of malaria from fourteen to twenty-two days from the time of inoculation, and that of actual malarial fever from ten to seventeen days. Despite these wide variations the percentage reduction in albumin in the eight patients was nearly the same, namely, from 30 to 40 per cent. One patient after three malarial inoculations experienced only four paroxysms and a total of one hundred ten hours of fever above  $100^{\circ}$  R. over a twenty-nine-day period, whereas a second patient after one inoculation experienced nine paroxysms and a total of seventy-one hours of fever above  $100^{\circ}$  R. over a ten-day period. Notwithstanding such differences the reduction in albumin in the former was 34 per cent and in the latter only 41 per cent. Tareev and Gontaewa,<sup>14</sup> however, contrary to our own findings, observed that the reduction in albumin in malarial infections paralleled the clinical severity. They observed more marked reductions in induced malaria than in the naturally acquired form; these were attributed to the more rapid recurrence of fever in the former type.

The rapidly induced anemia is a prominent feature of malaria, and one questions whether this factor plays any part in the low serum albumin levels obtained. Low serum albumin values have been found in anemias, both primary and secondary, but have been attributed to the accompanying malnutrition.<sup>4</sup> These anemias, however, are of a chronic type. The fall in albumin during malaria cannot be attributed to red cell destruction, since both Láng<sup>15</sup> and Bodansky and co-workers<sup>16</sup> found no change in the serum albumin of dogs in which anemia was produced by the administration of phenylhydrazine and acetyl phenylhydrazine, respectively.

The liver has been considered by some as the main source of albumin and the source of much of the globulin.<sup>17</sup> It is the sole source of fibrinogen. It has been shown that infections impair the manufacture of plasma proteins in the liver, despite the fact that protein intake may be maintained.<sup>18</sup> This may well explain the marked reductions in serum albumin (37 and 39 per cent) in two patients who exhibited a "ravenous appetite" throughout malaria yet showed as marked a reduction in albumin as a third patient whose food intake was poorer and whose weight loss was greater (10 pounds in contrast to 4.5 and 2.5 pounds). It does not explain the increases in globulin which were obtained at these times unless one predicates as Loeb<sup>2</sup> has that albumin and globulin are formed by different cells in the liver. Experimental observations reveal that only a small portion of protein is stored in the liver as preformed plasma protein.<sup>17</sup> Thus, not only is the liver unable to synthesize new plasma protein from exogenous material, i.e., food, because of an infection, but it is also unable to convert its stored protein into plasma protein because of a disturbance in the plasma protein-forming mechanism of the liver. The rapid rise in albumin practically immediately after the malaria was terminated, and at a

time when food intake remained essentially unchanged, would tend to verify the importance of infection in impairing serum protein production in the liver. The absence of an infectious process in fever induced by typhoid vaccine and artificial methods may well explain the slight and insignificant fluctuations in albumin which occurred during these types of therapy. In support of this is the finding of a marked reduction in albumin in typhoid fever,<sup>1</sup> though the very limited food intake at this time is an important factor not to be overlooked.

The low serum albumin values of severe infections have been attributed by some to the accompanying malnutrition,<sup>4</sup> i.e., insufficient protein intake. Continuous wasting due to malnutrition causes a steady fall in albumin.<sup>19</sup> It is difficult to evaluate true weight loss in patients undergoing malarial therapy, since subclinical fluid retention may well have occurred during its course because of low albumin values.<sup>6</sup> The weight losses obtained ranged from 1 to 10 pounds and, though they may indicate insufficient total calorie intake, they do not imply an inadequate protein intake. One patient showed a weight gain of 2.5 pounds during malarial therapy at a time when albumin had been reduced 34 per cent, from 4.82 Gm. to 3.23 Gm. No clinical evidence of edema was seen. It is possible that the protein intake of our patients, adequate for the patient when in normal health, was insufficient during malarial fever to supply the increased protein demands when fever was present. It does not appear logical to attribute the rapidly falling albumin during malaria to malnutrition, since the fall in albumin appeared early and even before the malarial fever occurred. This is well shown by the progressive fall in albumin from 4.82 to 4.01 Gm. in one patient over a thirteen-day afebrile incubation period, and the rapid fall in albumin from 5.1 to 4.3 Gm. in a second patient three days after malaria inoculation and one day before fever occurred. Sufficient protein storage in the liver and muscles should be present and readily available at this time to supply the body's needs,<sup>17</sup> especially since the dietary intake of each patient prior to and during the incubation period of malaria had been adequate. The albumin values of the eight patients were within normal limits prior to the induction of the malaria, with the exception of that of one patient who showed a low, unexplained level of 3.67 Gm. Little or no loss of serum albumin occurred in our patients by way of the kidney, since urine examinations infrequently revealed more than a very slight trace of albumin, and kidney function is relatively normal in uncomplicated malaria.<sup>20</sup>

The increases in globulin as a result of therapeutic fever were most marked during malaria, less marked during typhoid vaccine fever, and least marked during artificial fever. An increase in globulin occurs frequently as a result of infections, and is said to parallel the severity of the infection.<sup>5</sup> The increase in globulin during infections has been considered as mainly responsible for the high plasma protein levels found at these times.<sup>21</sup> A close association of certain antibodies with particular globulin fractions has been noted,<sup>2, 4</sup> as is seen by an increase in globulin following the subcutaneous injections of mixed typhoid vaccine. The destruction of red blood cells, as by the production of experimental anemias in dogs, has also resulted in an increase in globulin.<sup>15</sup> The greater increases in globulin during malaria may, therefore, be attributed to the presence of an infection and the destruction of red blood cells. Since

the reticulo-endothelial system participates in the regeneration of globulin,<sup>21</sup> one wonders whether stimulation of this system, said to occur during malaria,<sup>22</sup> may also play some part.

The fibrinogen level during health is relatively constant for any one individual and is not altered by short periods of fasting or by food ingestion.<sup>23</sup> Injury to the liver causes the blood fibrinogen to fall rapidly, the degree of fall paralleling somewhat the severity of liver injury. Regeneration and repair of liver tissue result in a return of the fibrinogen values to normal.<sup>17</sup> Injury to other tissues (as in infections) in the absence of liver injury is followed by an increase in fibrinogen.<sup>17, 23</sup> Fibrinogen values obtained on our patients imply that liver damage occurs as a result of malaria. Values fell considerably, even during the incubation period of malaria, in one patient from 520 mg. to 260 mg. Fibrinogen values obtained on this latter patient during malarial fever remained much below the prefebrile level. The falling values obtained on two additional patients as the malarial fever recurred also point to increasing impairment of liver functions. Clifford has stated that malaria always results in liver damage.<sup>24</sup> Studies of liver function following malarial therapy at this clinic indicate that liver damage of a temporary nature does occur.<sup>25</sup> It is significant that the course of the falling albumin during malaria did not differ in the three patients with probable liver damage from the remaining 5 patients who exhibited no significant reductions in fibrinogen. The rapid rise of albumin following the termination of the malaria would indicate that plasma protein regeneration occurs rapidly after the termination of the infection regardless of the fibrinogen values and probable liver damage. It would also tend to stress the importance of the infectious process per se in interfering with the production of albumin. The increases in fibrinogen as a result of typhoid vaccine fever have been observed by others.<sup>23</sup> It should be stressed, however, that fever per se is probably not the cause of the abnormally increased fibrinogen found in many febrile diseases.<sup>23</sup> Increases in fibrinogen occurred in our patients even during the incubation period of malaria when no fever had occurred. The fall in fibrinogen from 380 mg. to 270 mg. in one patient after eight artificial fever treatments (Chart 3) is difficult to explain. This value was obtained at a time when albumin also had fallen to its lowest level. Though liver function studies have not been carried out in this clinic during artificial fever therapy, occasional instances of jaundice have been observed. Significant pathologic changes in the liver have been found when fever therapy has terminated fatally.

#### CONCLUSIONS

1. Fever produced by inoculation malaria results in marked changes in the plasma proteins consisting of a rapid and marked drop in albumin, and fluctuations in globulin and fibrinogen. Termination of malarial fever by quinine is followed by a rapid return to normal values.

Fever produced by typhoid vaccine and artificial means (inductotherm) causes only slight fluctuations in the plasma protein fractions.

2. The more marked reduction in albumin during malaria may be due in part to the greater amount and the more rapid recurrence of fever. The in-

fectious process per se present in malaria is probably the most important factor interfering with the synthesis of albumin in the liver even though dietary intake appears adequate.

3. The increases in globulin are greater during malaria than during fever induced by typhoid vaccine or artificial means. The presence of an infectious process and the destruction of red blood cells during malaria account for the greater increases in globulin.

4. Fibrinogen is increased during malaria and typhoid vaccine fever. The progressive fall in fibrinogen, which occasionally occurs during malaria, indicates probable liver damage.

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# LABORATORY METHODS

## GENERAL

### "ELECTROCARDIOGRAPHIC ANAGRAMS,"\*

#### A METHOD OF TEACHING ELECTROCARDIOGRAPHY

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THE model described in this report has been used with considerable success in teaching electrocardiography in a simple logical manner. In principle it consists of cards of uniform sizes on which portions of P, Q, R, S, and T complexes are drawn to scale in such a fashion that the cards may be joined together or superimposed one upon another to form any of the more common electrocardiographic patterns. If only one portion of the electrocardiogram is changed at one time, the student soon learns to recognize the abnormality and to interpret its significance in a logical fashion. This method eliminates the necessity of textbook descriptions or patterns; it enables the student to lose some of his fear of electrocardiograms; and it allows him to test himself by creating characteristic patterns.

#### DIRECTIONS FOR PREPARING MODEL

Models of any size can be made. For classroom work, electrocardiographic complexes drawn to a scale of  $\frac{1}{2}$  inch for each millimeter of amplitude or each 0.4 seconds of duration have been found most serviceable; these are drawn on cards 10 inches high and 3, 6, or 12 inches wide. A complete set consists of 90 cards; 16 cards, 10 by 12 inches; 14 cards, 10 by 6 inches; and 60 cards, 10 by 3 inches.

*Materials.*—The following materials are necessary; 38 pieces of heavy white noncurling cardboard, each measuring 10 by 12 inches; two sheets of transparent cellulose acetate, approximately 10 by 12 inches; ruler; pencil; soft wax pencil; black poster paint; drawing pen; camel's hair paint brush; scissors; sharp knife or razor blade.

*Method.*—With a pencil draw two lines, 1 inch apart and 12 inches long, through the center of each card; this represents the electrocardiographic base line, or a string shadow 2 mm. wide. On four of these cards this base line is inked in. Two cards are left intact (Fig. 1 *a*). One card is cut in two to form two models, 10 by 6 inches each (Fig. 1 *b*); one is cut to form four cards, 10 by 3 inches (Fig. 1 *c*). A black ink dot is placed in the upper left corner of each of these and subsequent cards to make possible rapid recognition of the upright position.

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*Normal Complexes* (Fig. 2).—Starting  $\frac{1}{2}$  inch from the left-hand margin of one card, draw a P-wave, one inch high and approximately  $1\frac{1}{2}$  inches wide. Beginning 3 inches from the left-hand margin, draw a QRS complex, 1.25 inches in width, the Q-wave should be approximately  $\frac{1}{2}$  inch in amplitude, the R- and S-waves 3 to 4 inches in amplitude. Beginning 6 inches from the left-hand margin, a T-wave is drawn  $\frac{3}{4}$  inch in amplitude and 2 inches wide; if desired the terminal limb of the T-wave can be drawn to dip  $\frac{1}{4}$  inch below the base line, returning to the isoelectric level, 9 inches from the left-hand margin. By following these directions the P-wave will be the upper limit of normal in amplitude; the T-wave will be the lower limit of normal in amplitude; the QRS complex and the PR-interval will be the upper limit of normal in duration (Fig. 2 a). One piece of cellulose acetate is superimposed on this drawing; the contour of the

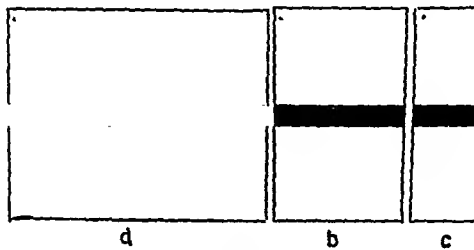


Fig. 1.

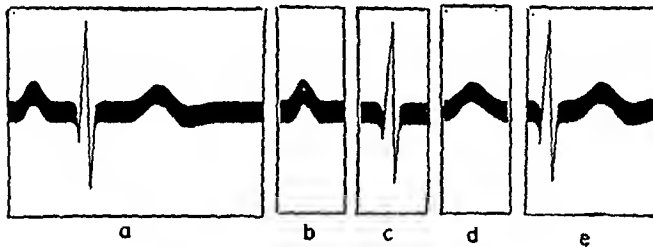


Fig. 2.

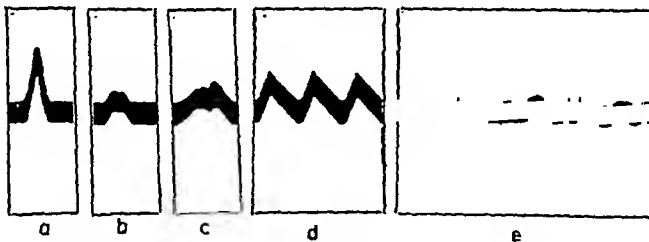


Fig. 3.

diagrammatic complex is traced with a wax pencil, and the resultant P-QRS-T-complex is cut out. This results in a templet, which can be used to draw normal complexes of identical characteristics. A total of six P-QRS-T-complexes are prepared from this templet, and the outlines are filled in with poster paint.

Lines are drawn on four cards to divide them into sections, 10 by 3 inches. By using the appropriate portions of this same templet, six P-waves (Fig. 2 b) four QRS complexes (Fig. 2 c) and six T-waves (Fig. 2 d) are drawn on 10 by

3 inch cards. With the same templet normal QRS-T complexes are drawn on four 10 by 6 inch cards to depict ST-segments of short duration (Fig. 2 e).

*Abnormal P-Waves.*—Six P-waves, 2 inches high (Fig. 3 a), and six notched P-waves (Fig. 3 b),  $\frac{3}{4}$  inch high, are drawn on 10 by 3 inch cards after preparing the appropriate templets. Four cards (10 by 3 inches) are prepared showing normal T-waves on which abnormal P-waves are partially superimposed (Fig. 3 c).

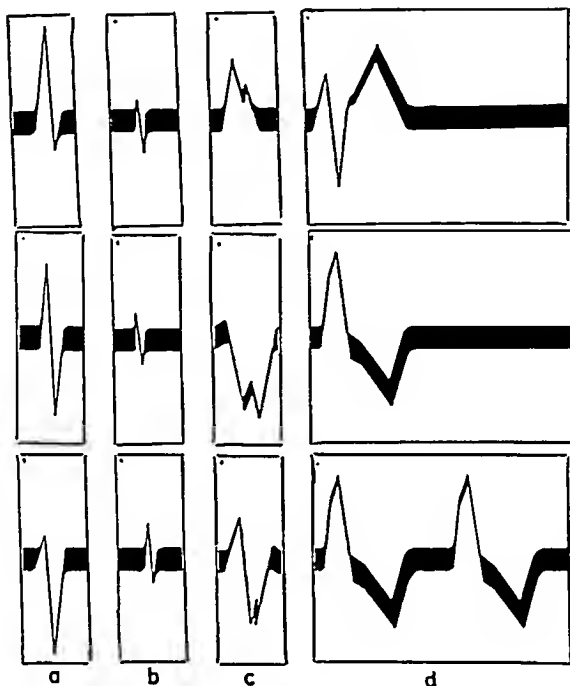


Fig. 4.

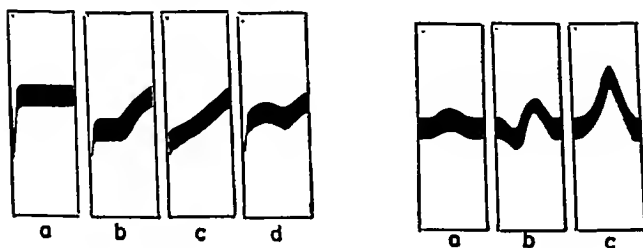


Fig. 5.

Fig. 6.

A flutter wave, 2 inches in duration, is drawn, and from the templet four cards, 10 by 6 inches, are prepared, each bearing three flutter waves (Fig. 3 d). By making the base line of three cards irregular, the f waves of auricular fibrillation are depicted (Fig. 3 e).

*Abnormal QRS Complexes.*—QRS complexes to show axis deviation are drawn on three cards (Fig. 4 a), 10 by 3 inches, as follows: On one card a large R-wave and a small S-wave are drawn, and a templet is made. By inverting and reversing the templet, a complex with a small R-wave and a large S-wave is

drawn on a second card. The third complex is drawn to represent an average of these two types of QRS complexes.

QRS complexes of low amplitude are demonstrated (Fig. 4 *b*) on three 10 by 3 cards. Each card represents one of the three standard leads. The complexes are drawn less than  $1\frac{1}{4}$  inches in width and less than  $3\frac{1}{2}$  inches in amplitude.

Bundle branch block (spreading, slurring, and notching) is represented on three 10 by 3 inch cards (Fig. 4 *c*); the major deflection is upright on one card, and downward in another card; the third card represents an average of the first two.

Premature ventricular contractions of two different designs are drawn on 10 by 3 inch cards (Fig. 4 *d*). The abnormal QRS-T complexes should begin at the left-hand margin of the card and occupy 4 to 5 inches; the remainder of the card serves to depict the compensatory pause. Using a templet drawn from one of these extrasystoles, three additional cards, each containing two ventricular premature beats, are drawn so that when these are joined together they illustrate a run of ventricular extrasystoles or ventricular tachycardia.

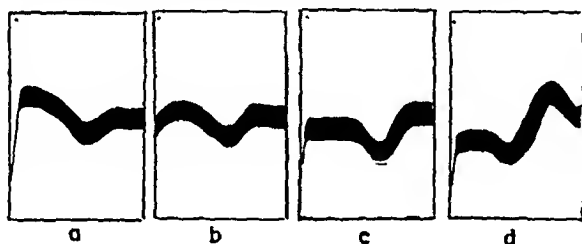


Fig. 7.

*Abnormal ST-Segments and T-waves.*—Four types of abnormal ST-segments are illustrated on 10 by 3 inch cards (Fig. 5). These are drawn so that the left-hand margin of each can be superimposed on the normal S-wave, and the right-hand margin can be superimposed on the normal T-wave. One card depicts an elevated ST-segment parallel to, and 1 inch above, the base line (Fig. 5 *a*). A second card depicts a depressed ST-segment, also parallel to the base line (Fig. 5 *b*). A third card begins 1 inch below the base line, and then runs diagonally up to reach the peak of the T-wave (Fig. 5 *c*). The fourth card illustrates coving (Fig. 5 *d*). Four T-waves,  $\frac{1}{2}$  inch high, are drawn on cards 10 by 3 inches (Fig. 6 *a*). One diphasic T-wave (Fig. 6 *b*) is drawn on another 10 by 3 inch card. Six T-waves, 3 inches in amplitude (Fig. 6 *c*), are drawn on 10 by 3 inch cards; these may be used to depict normal T-waves of high amplitude or T-waves on which P-waves have been superimposed.

Four 10 by 6 inch cards, illustrating combinations of abnormal ST-segments and T-waves, are drawn. One depicts an elevated ST-segment and inverted T-wave (Fig. 7 *a*); another, coving with an inverted T-wave (Fig. 7 *b*); a third, a depressed ST-segment, with an inverted T-wave (Fig. 7 *c*); and a fourth, a depressed ST-segment with diphasic T-wave (Fig. 7 *d*; digitalis T-waves).

To demonstrate these cards a stand can be made according to Fig. 8. This stand also serves as a container for the cards when they are not in use.



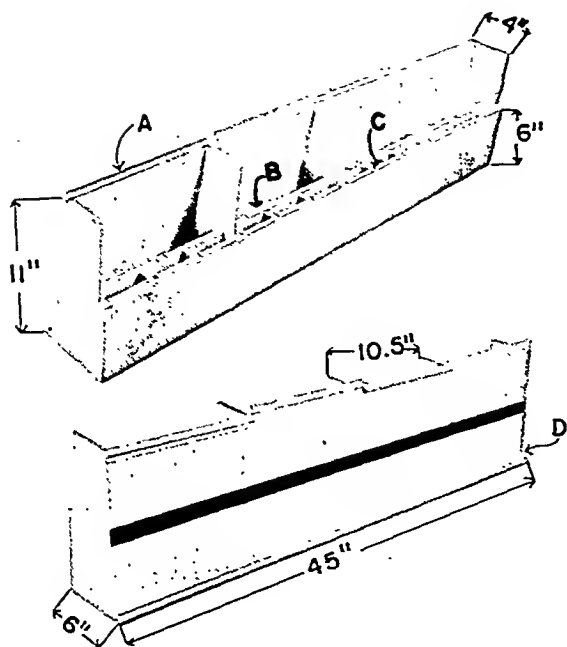


Fig. 8.—Viewing stand for demonstrating electrocardiographic patterns. (This viewing stand can be made of  $\frac{1}{2}$  inch stock.) A, Hinged molding to hold upper edge of cards in place; B, container for large cards,  $10\frac{1}{2}$  by  $2\frac{1}{2}$  inches; C, container for small cards,  $3\frac{1}{2}$  by  $1\frac{1}{2}$  inches; and D, grooved molding to hold lower edge of cards in place.

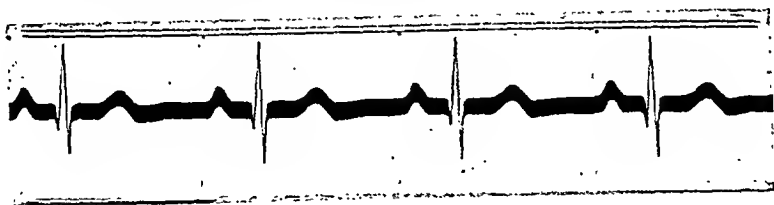


Fig. 9.

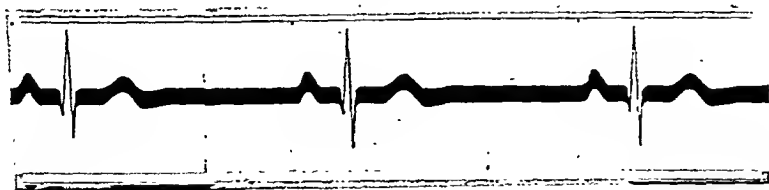


Fig. 10.

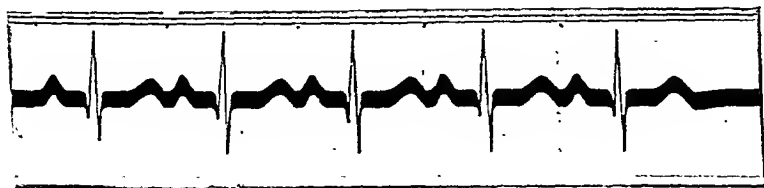


Fig. 11.

## METHOD OF USE

In use, normal sinus rhythm is first illustrated by joining end-to-end four normal P-QRS-T complexes (Fig. 9). Arrhythmias arising from the sino-auricular node are then illustrated: sinus bradycardia, by separating three normal P-QRS-T complexes (Fig. 10); sinus tachycardia, by partially superimposing the complexes so that five occupy the same horizontal area (Fig. 11).

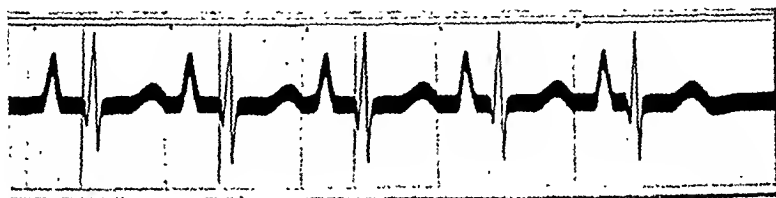


Fig. 12.

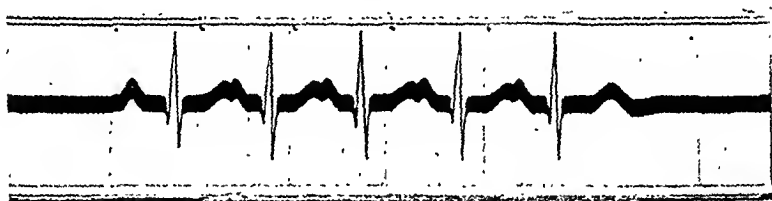


Fig. 13.

Abnormalities of the P-waves are next illustrated. Auricular tachycardia is depicted by superimposing an abnormal P-wave on each of the normal P-waves of the sinus tachycardia (Fig. 12), or by further superimposition of the normal complexes so that the P-waves cover the T-waves, and then superimposing the combined P-T waves (Fig. 13), large T-waves, or inverted P-waves. In a similar fashion abnormalities of the PR-interval (varying degrees of block) are illustrated, then abnormalities of the QRS complexes, the ST-segment, and the T-waves. Practically all the electrocardiographic patterns, which one encounters clinically, can be created by this method.

## SUMMARY

The model described consists of units of electrocardiographic complexes prepared in such a fashion that they can be joined together so as to illustrate diagrammatically most of the common electrocardiographic patterns. The model has proved to be of considerable value in teaching beginners how to interpret electrocardiograms.

## SIMPLE METHOD OF EVALUATING BLOOD PLATELETS\*

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THE procedure for accurately seeing, identifying, and counting the blood platelets has become more important with the discovery that splenectomy cures a high percentage of patients afflicted with idiopathic purpura hemorrhagica. The methods used and recommended by most textbooks are based on dilution and counting with the hemacytometer. The most popular diluting fluid is that of Wright and Kinnicutt, though the fluids recommended by Rees and Ecker, and Guy and Leake are also extensively used. The Fonio method, so highly recommended by the German school, which uses the magnesium sulfate as a diluent before spreading the blood on the slide for a smear, also has been extensively used. After several years of trial with the dilution methods and the Fonio method with students in classes of Laboratory Diagnosis, it is our opinion that the following method is the simplest and least time-consuming for the accurate identification and counting of blood platelets.

The solution used is a 1 per cent brilliant cresyl blue in absolute alcohol. A drop of the solution is placed on the edge of a slide (thoroughly cleaned and flamed), and spread out in a manner similar to spreading blood for a blood smear. The solution evaporates readily, leaving a thin coat of stain. An ear puncture is made, and a drop of blood the size of a black pinhead is placed on a thoroughly cleaned cover slip and overlaid on the stained slide. A red blood cell count is also drawn at the same time. The size of the drop must not be too large so that it spreads out uniformly filling the cover slip area with a thin film of blood. The platelets take the brilliant cresyl blue stain and appear as pale blue bodies floating between the less colored red blood cells. Variations in shape and size are also readily noted in the preparation. A total of 1,000 red blood cells are counted on the film, and the number of platelets in the same area is noted. From the value of the red blood count, the platelet count is calculated. For example, if in counting 1,000 red blood cells, we encounter 75 platelets, and the patient's blood count is 4,750,000 red cells, by multiplying the first four figures of the red count with the number of platelets in 1,000 red cells we get the platelet count, which in the above case would be 75 times 4,750, or 356,250 platelets.

This method offers the advantage that reticulocytes may also be counted on the same preparation. If kept in a Petri dish lined with moistened filter paper, it may be kept several hours without the platelets disintegrating. After first observing the moist preparation, the cover slip may be removed with light pressure and counterstained with Wright's stain or May-Grünwald-Giemsa for further observation of the platelets and a differential count.

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## A SHAKING DEVICE USED IN THE COLLECTION OF BLOOD FOR TRANSFUSION\*

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A SIMPLE, noiseless and relatively inexpensive shaking device (Fig. 1) has been developed in the Blood Bank at the John Gaston Hospital. The power is supplied by a small electric motor from a portable vietrola. The back and forth motion is obtained by a shaft attached to an eccentric disk. The carriage for the collecting bottle is swung from two metal supports 12 inches in length.

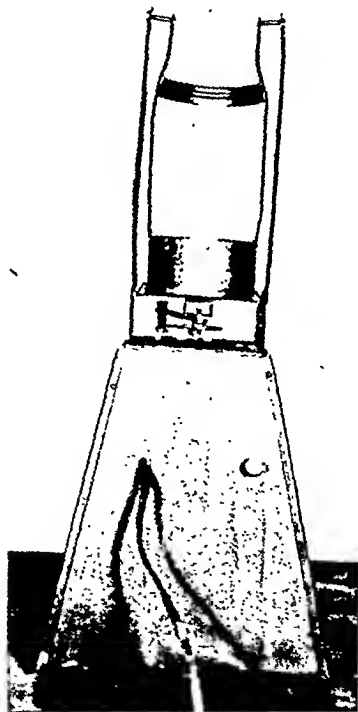


Fig. 1.—A shaking device used in the collection of blood for transfusion.

The fulcrum is placed at the level of the top of the bottle so that motion and vibration at this point are minimal. The carriage is open at the top so that the collecting bottles can be easily inserted and removed. The height of the instrument is 23 inches, the width at the base 10 inches, and the width at the top 5 inches.

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The shaking device has been used for the collection of approximately 2,000 flasks of blood for transfusions and has proved to be entirely satisfactory. The assistant is spared the tiresome and awkward task of keeping the flask of blood in motion during the three to fifteen minutes required to collect 500 c.c. of

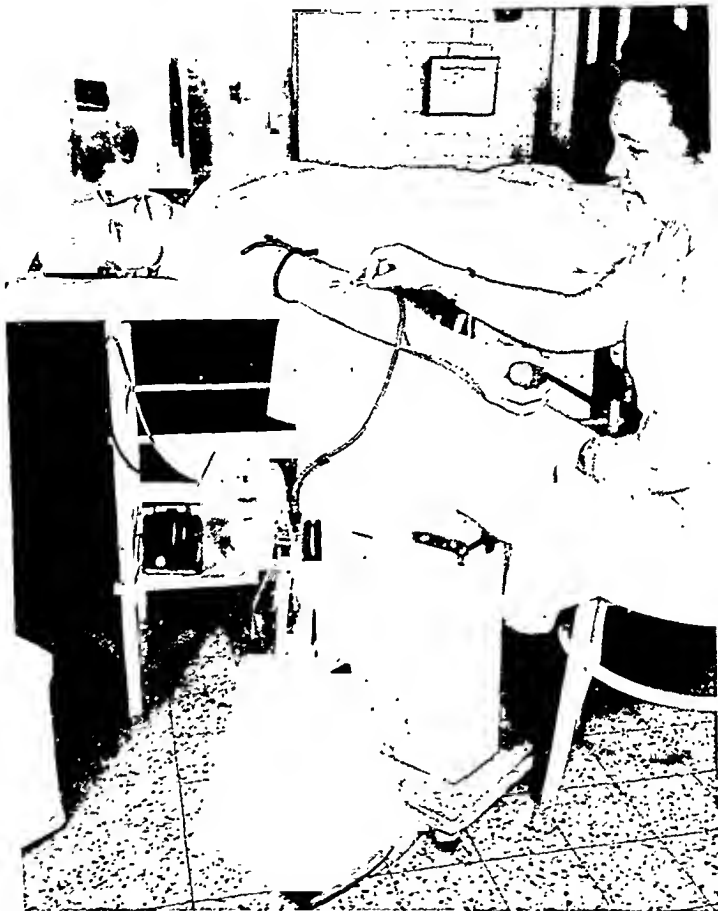


Fig. 2.—The collection of blood from a donor by the closed citrate method using "the shaker."

blood. With the shaker it is possible for one person to collect the blood unassisted. The gentle agitation furnished by this instrument is adequate to prevent coagulation. In our routine collections the blood is simply allowed to run downhill without the use of negative pressure, but if negative pressure is desired, the suction pump may be connected as in Fig. 2.

The instrument was built by the students at the William R. Moore School of Technology under the direction of Mr. J. M. Smith.

## THE RELATIONSHIP OF THE PLASMA PROTEINS TO THE CORRECTED SEDIMENTATION RATE\*

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MANY observers have demonstrated a suggestive relationship between the red blood cell sedimentation rate and the fibrinogen content of the plasma, and some have concluded that the fibrinogen content determines the sedimentation rate.<sup>1, 2</sup> Others,<sup>3, 4</sup> however, have failed to observe any such causal relationship and have found no correlation between the sedimentation rate and any of the plasma proteins. Marked changes in the sedimentation rate occur even without any changes in the plasma proteins.<sup>4</sup> Increases in globulin and extreme degrees of lipemia are also known to affect the sedimentation rate.<sup>5</sup>

Repeated determinations of the plasma protein fractions and the sedimentation rate on patients undergoing therapeutic fever for central nervous system syphilis have afforded an opportunity to review again the problem of the relationship if any between the sedimentation rate and the plasma proteins.

### MATERIAL AND METHODS

Five male patients with general paresis, ranging from 39 to 52 years in age, were hospitalized for fever therapy. The five patients were given the following therapy: tertian malaria to two patients; tertian and quartan malaria to one patient; tertian malaria and typhoid vaccine fever to one patient; artificial fever and malaria to the fifth patient.

Venous blood samples were obtained without stasis and with the patient in a fasting state. In most instances the patient was afebrile. Potassium oxalate, 2 mg. per cubic centimeter of blood, was used as the anticoagulant for the determination of the plasma proteins.

The total protein was obtained by the determination of the total nitrogen by a modified micro-Kjeldahl<sup>6</sup>; the nonprotein nitrogen by the method of Folin and Wu<sup>7</sup>; the albumin and globulin by the sodium sulfate method of Howe<sup>8</sup>; and the fibrinogen by precipitation as fibrin by the method of Cullen and Van Slyke.<sup>9</sup> In the last series of studies the fibrinogen was precipitated as above,<sup>9</sup> and the nitrogen content of the fibrin was determined directly.

The sedimentation rate of whole blood was determined by the method of Rourke and Ernstene<sup>10</sup> and corrected for the hematocrit readings by their correction chart. Dry oxalate mixture, 6 mg. of crystalline ammonium oxalate and 4 mg. of crystalline potassium oxalate for 5 c.c. of blood, was used as the anticoagulant.

\*From the Boston Psychopathic Hospital.  
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The sedimentation rate of defibrinated blood was determined by the method of Ham and Curtis.<sup>2</sup> Fibrinogen was removed by rotating 5 c.c. of blood together with 12 small glass beads in an Erlenmeyer flask for from ten to fifteen minutes, and then filtering through two layers of gauze. The defibrinated blood was then mixed, and the sedimentation rate was determined as with whole blood. Because of the slow settling, readings were made over a period of three hours. Corrections were made for the hematocrit according to the charts of Ham and Curtis,<sup>2</sup> and Rourke and Ernstene.<sup>10</sup>

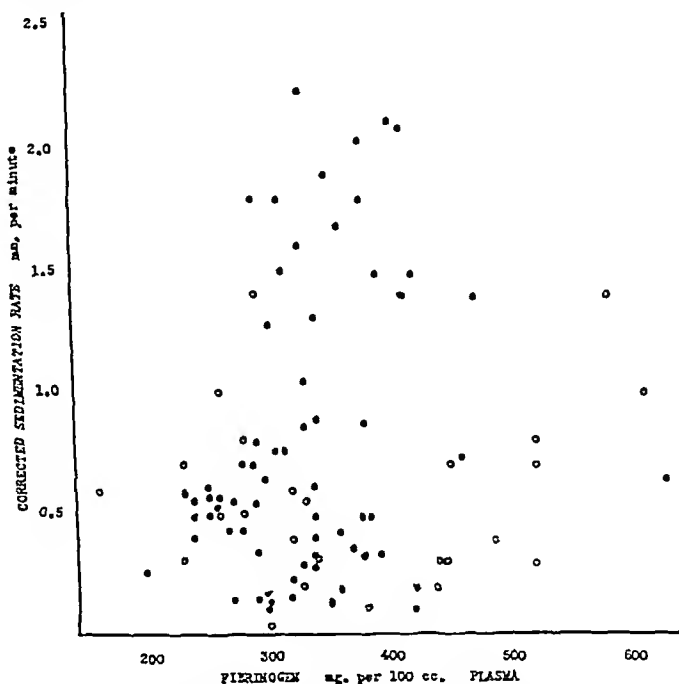


Chart 1.—○ Fibrinogen by difference. • Fibrinogen by direct method.

#### RESULTS

Ninety-two sedimentation rates were obtained during malaria, typhoid vaccine, and artificial fever therapy. Taken as a whole no linear relationship could be found between the corrected sedimentation rates and the fibrinogen values (Chart 1). The sedimentation rate varied more than 1,500 per cent for the same fibrinogen value. Fibrinogen values also varied widely for similar sedimentation rates, as for example, sedimentation rates of 0.60 mm. and 0.65 mm. per minute for fibrinogen values of 160 mg. and 630 mg., respectively. The sedimentation rates also bore no absolute relationship to albumin, albumin-globulin, or globulin-fibrinogen ratios.

It was observed frequently that when the more rapid sedimentation rates could not be explained on the basis of fibrinogen values alone, the accompanying globulin values were close to the upper limit of normal, 3 Gm. per 100 c.c. or above. However, wide variations in the sedimentation rate, as much as 2,200 per cent, occurred even for similar globulin values (Chart 2).

The sedimentation rates of each patient revealed a suggestive linear relationship with fibrinogen values in only two of the five patients, one during malarial therapy and the second during artificial fever therapy. Changes in the globulin fraction during treatment appeared less marked in the two patients than in the others. The subsequent administration of tertian malaria to the patient who had received artificial fever therapy resulted in more scattered sedimentation rates for nearly similar fibrinogen values (Chart 3). Globulin values, though higher at this time than during artificial fever therapy, were still within normal limits.

An attempt was made to evaluate the importance of globulin in the determination of the sedimentation rate by obtaining 54 defibrinated blood sedimentation rates on three patients during malarial therapy, and on one of this group during a course of typhoid vaccine fever. The removal of fibrinogen did not prevent an increase in the sedimentation rate, but the increases when present were less marked than those of whole blood, i.e., fibrinogen was not removed. It will be observed (Chart 2) that the sedimentation rates with and without the presence of fibrinogen follow about the same pattern when compared with the globulin values. No correlation was found between the defibrinated sedimentation rates and globulin values when the latter were 2.8 Gm. per 100 c.c. or less. Above this level, however, a suggestive linear correlation was present, the defibrinated sedimentation rates showing marked increases with only slight increases in globulin.

#### DISCUSSION

Our findings when taken as a whole reveal little correlation between the corrected whole blood sedimentation rate and fibrinogen values in therapeutic malaria and typhoid vaccine fever. The corrected sedimentation rates obtained during artificial fever therapy of one patient and malaria therapy of a second patient bore a suggestive linear relationship to fibrinogen values. Changes in the fibrinogen, globulin, and red blood cells are quite marked during malaria, less marked during typhoid vaccine fever, and least marked during artificial fever. It is probable that a better relationship between the sedimentation rate and fibrinogen may exist when changes in the plasma proteins and red blood cells are not rapid or marked. Ropes and co-workers,<sup>4</sup> however, have observed marked changes in the sedimentation rate in both acute and chronic infections even without any change in the concentration of the plasma proteins. No absolute correlation was found by these workers between the sedimentation rate and any of the plasma protein fractions.

The sedimentation rates obtained with whole blood and defibrinated blood samples show the importance of globulin. Regardless of fibrinogen levels, globulin values of 2.9 Gm. per 100 c.c. and above were frequently accompanied by rather marked increases in the sedimentation rates of whole blood but less pronounced in the defibrinated samples. Defibrinated sedimentation rates with globulin values less than 2.9 Gm. per 100 c.c. ranged mostly from 0.01 to 0.2 mm. per minute (Chart 2) and were frequently above the normal range of 0.01 to 0.03 mm. per minute.<sup>2</sup> Defibrinated sedimentation rates increased consider-



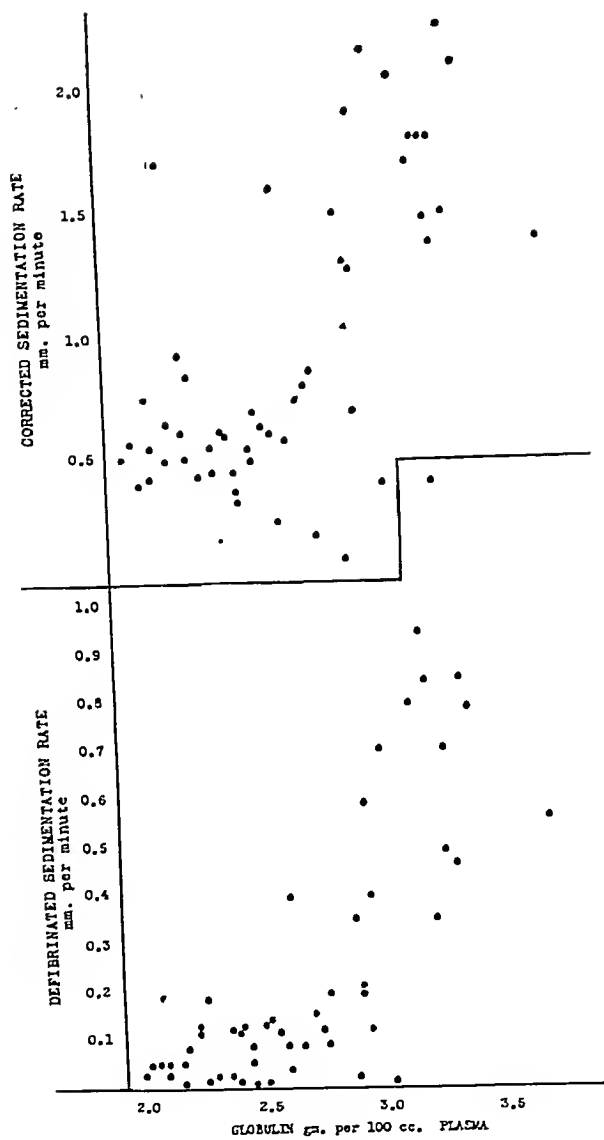


Chart 2.

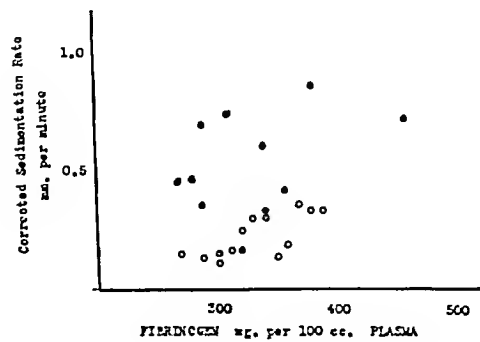


Chart 3.—• Malaria. ○ Artificial fever.

ably up to 0.95 mm. per minute, when globulin levels exceeded 2.9 Gm. per 100 c.c. Since it has been shown that albumin does not increase the sedimentation rate, but may even slow it,<sup>4</sup> the increases in the defibrinated sedimentation rate must be attributed to changes in the globulin fraction, quantitatively or qualitatively, or to some other unknown factor. Ham and Curtis<sup>2</sup> have also noted marked elevations in the defibrinated sedimentation rate when serum globulin was abnormally increased. They have found, too, a rough correlation to exist between the concentration of serum globulin when above 3 Gm. per 100 c.c. and the defibrinated sedimentation rate provided lipemia is not present.

Other factors which may affect the sedimentation rate and account for its lack of correlation with the fibrinogen values obtained on our patients are the following: the mean corpuscular volume of the red blood cells, the presence of a lipemia, and liver pathology.

Changes in the mean corpuscular volume of the red blood cells are known to affect the sedimentation rate and are not taken into account when the corrected sedimentation rate is determined.<sup>2</sup> The mean corpuscular volume of the blood during therapeutic fever showed fluctuations as great as -12 to +17 cubic microns; these were especially marked during malarial therapy. The presence of a lipemia increases the sedimentation rate, but determinations on a number of our own patients and observations of others<sup>11</sup> reveal a reduction and not an increase of the cholesterol content of the blood in malaria. We do not know whether changes in the cholesterol occur during fever induced by typhoid vaccine or by artificial means. Gilligan and Ernstenc<sup>4</sup> have stated that in liver diseases factors other than fibrinogen, among them globulin, may play an important role in the determination of the sedimentation rate. They, and others,<sup>2</sup> have observed a rapid sedimentation rate in acute hepatitis and cirrhosis of the liver. It is the feeling of Clifford<sup>12</sup> that the liver is damaged in malaria and liver function tests carried out in this clinic show occasional but transient liver damage. In addition progressive reductions in fibrinogen have been observed during therapeutic malaria which would tend to corroborate this. However, rapid sedimentation rates have been obtained on our patients even when fibrinogen has fallen to the lower limit of normal (190 mg. per 100 c.c. of blood) and globulin has remained within normal limits. This does not eliminate the possibility of qualitative changes in the globulin fraction.

An attempt has been made by Ropes and co-workers<sup>4</sup> to explain the inconsistencies between sedimentation rates and fibrinogen values. These workers attribute the variations in the sedimentation rate to modifications in the physical state of the plasma colloids with consequent changes in the electric charges on the proteins and red blood cells. Changes in the colloidal state and increases in the sedimentation rate may thus occur without any change in fibrinogen or globulin values.

#### CONCLUSIONS

1. Corrected whole blood sedimentation rates and plasma protein values were determined repeatedly on five patients undergoing therapeutic fever (malaria, typhoid vaccine, and artificial fever) for general paresis.

2. No absolute correlation could be found between fibrinogen, albumin, albumin-globulin, or globulin-fibrinogen ratios on the one hand, and the corrected sedimentation rate on the other.

3. The corrected sedimentation rate was increased in most instances when globulin values rose to the upper limit of normal (3 Gm. per 100 c.c.) and above, regardless of fibrinogen levels.

4. Other factors which may influence the sedimentation rate during therapeutic fever are changes in the mean corpuscular volume of the red blood cells and products of probable liver damage.

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## DEHYDRATION IN EMBALMED SPECIMENS EXPOSED TO AIR

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ONE of the claims frequently made for glycerin and other polyhydric alcohols as embalming fluid ingredients, is that they retard the loss of water from the subject. We have made a study of this claim from both theoretical and practical viewpoints.

A fresh specimen or body may be regarded as a hydrophylic colloidal system, containing about 60 per cent water. This water content is composed of two components: free water, which provides the proper life environment, and bound water, that which is bound to the biocolloids of tissue through coordinate valence linkages. About 25 per cent of the total water may be in the latter form.<sup>1</sup> Such bound water is less susceptible to evaporation loss than the free water. Moreover, as a result of incipient putrefaction, the complex colloidal structures become degraded and begin to lose the power of binding water. Formaldehyde fixation causes profound changes in protein structures, converting them into insoluble inert products with little water-binding power. As a result of both of these processes, it is logical to regard most of the water found in dead animal bodies as free water. As such, the water is as free to evaporate as is the water in an open dish. The prevention of this loss of water is important in the preservation of museum specimens and bodies which must be preserved for a long time. Water loss from bodies placed in sealed glass top caskets presents a specially troublesome problem.

An obvious method of reducing the evaporation is to dissolve additional solutes in the injection fluid. All nonvolatile solutes reduce the vapor pressures of solutions, in accordance with the law of Raoult. Those which are hygroscopic and form hydrates are most efficient as evaporation retarders. In the tobacco industry<sup>2</sup> glycerin, ethylene glycol, and diethylene glycol have been successfully added in order to maintain the moisture content of tobacco under storage. It has seemed natural to expect that these compounds would also prevent the drying out of animal remains even though larger concentrations of water occur in them.

An inspection of the vapor pressure curves of solutions of these compounds shows that this expectation cannot be realized to any great extent. In the case of glycerin (Fig. 1) a solution must have a concentration of 80 per cent by weight in order to remain in equilibrium with air having a relative humidity of 50 per cent. If the relative humidity is even 95 per cent, so that evaporation is greatly retarded, the solution must still contain 33 per cent of pure glycerin. Thus a normal adult body would need to have injected into it over 2.5 gallons

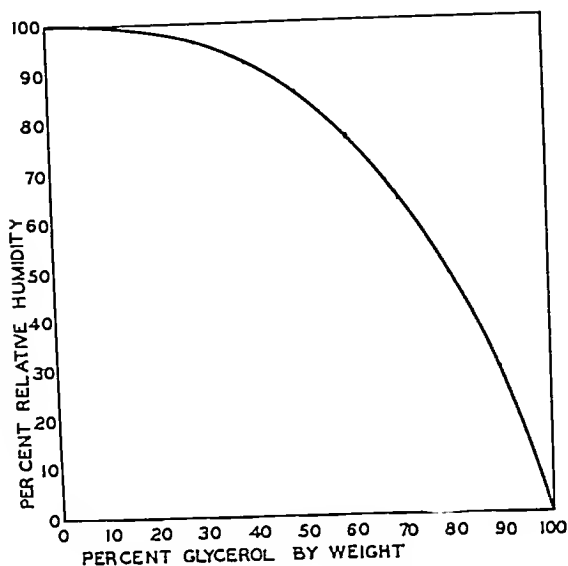


Fig. 1.—Relative humidity over aqueous glycerol solutions.

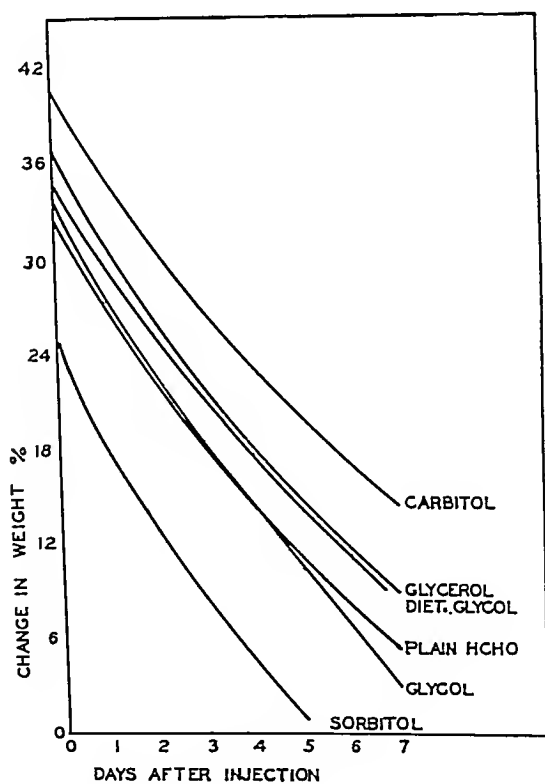


Fig. 2.—Water loss from arterially injected specimens.

of pure glycerin in order to prevent water loss from occurring upon exposure to air at 95 per cent relative humidity. A greater amount of another compound is required if its molecular weight exceeds that of glycerin. In the case of glycol, which is the only nonvolatile alcohol having a molecular weight less than that of glycerin, about two gallons would be required. It is obviously impossible to inject these huge amounts of viscous liquids and we may, therefore, conclude that it is impossible to prevent the gradual loss of water from a body by adding these compounds to the fluid used in injecting it. None of the commercial fluids we examined privately contained over 6 per cent of glycerin or similar product when diluted according to directions, and it is evident that these would have only a slight effect in delaying the loss of water from a body.

#### EXPERIMENTAL

We have measured the desiccation losses of a large number of aridly stored hog kidneys which had been arterially embalmed with aqueous 2 per cent formaldehyde solutions containing various concentrations of nonvolatile organic solutes. Each kidney was injected through the renal artery with an amount of fluid equal to 37.5 per cent of its fresh weight. After injection the renal artery, vein, and ureter were tied off, and the kidneys were stored at room temperature in large containers over fused calcium chloride. The control kidneys were injected with plain 2 per cent formaldehyde solution, while the fluid used for the others contained in addition, 2.5, 5, and 10 per cent of the following compounds: glycerin, ethylene glycol, diethylene glycol, carbitol, and sorbitol. The kidneys were weighed every day to determine the loss of water from them.

Figs. 1 and 2 show the weight changes in the embalmed material with time in the case of the 10 per cent additions. All the plotted values represent average values on several specimens. Except for minor displacements up or down due to uncontrollable differences in the biological material, the graphs have identical slopes and shapes, and are nearly superimposable. Only slight differences in water loss are found, and we may conclude that very little can be done in reducing the water loss from embalmed bodies unless impracticably large amounts of hygroscopic agents are injected.

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# THE USE OF SS (SHIGELLA-SALMONELLA) AGAR FOR THE ISOLATION OF FLEXNER DYSENTERY BACILLI FROM THE FECES\*

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SS (SHIGELLA-SALMONELLA) agar is a new culture medium for the isolation of microorganisms of the Shigella, Salmonella, and Eberthella groups from the feces. The medium is of the "selective" type, i.e., it is designed to favor the growth of these pathogens, but to inhibit the coliform bacteria. This selective inhibition of the fecal bacteria enables very large inocula of feces to be used, with a consequently greater chance of cultivating the pathogens when they are present in small numbers. Colonies of pathogens are colorless, whereas any colonies of the nonpathogenic lactose fermenters that develop appear red. The medium is similar in purpose to desoxycholate-citrate agar,<sup>1</sup> the value of which is well-established,<sup>2</sup> but differs considerably from it in composition.†

Mayfield and Gober<sup>3</sup> have furnished the only report yet available on the use of SS agar. These authors examined more than one thousand stool specimens, of which 101 were positive for *E. typhosa*, and 171 were positive for *Sh. dysenteriae*, chiefly of the Flexner group. Each stool specimen was cultured on both desoxycholate-citrate agar and SS agar, and the results on the two media were compared. Of the 272 specimens containing typhoid or dysentery bacilli, 262 were positive on SS agar, and 249 were positive on desoxycholate-citrate agar. Cultures were positive on SS agar alone in 23 specimens, and on desoxycholate-citrate agar alone in 10 specimens. These authors concluded that SS agar was as efficient as desoxycholate-citrate agar for the isolation of dysentery bacilli. They also noted that differentiation between the fecal and pathogenic colonies was more clearly defined on the SS agar, and that the medium was economical.

We have recently had the opportunity to employ SS agar in the bacteriologic study of a small outbreak of Flexner dysentery. Seventeen clinical cases of dysentery were observed, nearly all of which were mild, although several pa-

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†SS agar is a product of the Difco Laboratories, Detroit, Mich. This medium and the others used in this study were purchased on the open market.

It has the following formula:

|                    |           |
|--------------------|-----------|
| Beef extract       | 5.0 Gm.   |
| Proteose peptone   | 5.0 Gm.   |
| Lactose            | 10.0 Gm.  |
| Bile salts         | 8.5 Gm.   |
| Sodium citrate     | 8.5 Gm.   |
| Sodium thiosulfate | 8.5 Gm.   |
| Ferric citrate     | 1.0 Gm.   |
| Agar               | 17.0 Gm.  |
| Brilliant green    | 0.33 mg.  |
| Neutral red        | 0.025 mg. |

Weights of ingredients are per liter. The formula for desoxycholate-citrate agar will be found in reference 1.

tients experienced a severe febrile reaction. Diarrhea persisted in most cases for from three to seven days. The stools contained much mucus, but were rarely bloody. There were no fatalities.

Bacteriologic examinations of the feces from patients and contacts were done in the diagnostic laboratory of the Presbyterian Hospital. This laboratory routinely uses for stool cultures desoxycholate-citrate agar and a nonselective medium, eosin-methylene blue agar. SS agar was introduced shortly after the outbreak began.

A total of 225 stool specimens were examined from patients and contacts. Dysentery bacilli of the Flexner group were isolated from each of the 17 clinical cases and from one of the contacts. Altogether, 70 of the 225 specimens were found to be positive on one or more of the three media. Of the positive specimens, 14 were cultured on eosin-methylene blue agar and desoxycholate-citrate agar. SS agar with either eosin-methylene blue agar or desoxycholate-citrate agar was used for 26 specimens. The remaining 30 specimens were cultured on all three media.

TABLE I

COMPARATIVE EFFICIENCY OF EOSIN-METHYLENE BLUE AGAR, DESOXYCHOLATE-CITRATE AGAR, AND SHIGELLA SALMONELLA AGAR FOR THE ISOLATION OF FLEXNER DYSENTERY BACILLI

| 70 POSITIVE SPECIMENS<br>COMBINATIONS OF MEDIA USED | RESULTS ON INDIVIDUAL MEDIA |                    |                    |                    |                    |                    |
|---|-----------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
|   | EMB AGAR                    |                    | DC AGAR            |                    | SS AGAR            |                    |
|   | NUMBER<br>POSITIVE          | NUMBER<br>NEGATIVE | NUMBER<br>POSITIVE | NUMBER<br>NEGATIVE | NUMBER<br>POSITIVE | NUMBER<br>NEGATIVE |
| EMB and DC, 14 specimens                            | 7                           | 7                  | 13                 | 1                  | -                  | -                  |
| EMB and SS, 20 specimens                            | 9                           | 11                 | -                  | -                  | 20                 | 0                  |
| DC and SS, 6 specimens                              | -                           | -                  | 5                  | 1                  | 6                  | 0                  |
| EMB, DC, and SS, 30 specimens                       | 10                          | 20                 | 25                 | 5                  | 29                 | 1                  |
| Total positive and negative on individual media     | 26                          | 38                 | 43                 | 7                  | 55                 | 1                  |
| Per cent positive on individual media               | 40                          |                    | 86                 |                    | 98                 |                    |

The results of cultures on the four combinations of media are given in Table I. Eosin-methylene blue agar yielded 26 positives out of 64 cultures in which this medium was used, an incidence of only 40 per cent. The cultures on desoxycholate-citrate agar were positive in 43 of 50 specimens, or 86 per cent. SS agar was positive in 98 per cent of cultures, only one specimen out of 56 being negative on this medium.

The data indicate clearly that a large proportion of the positive specimens would have been considered negative if the nonselective medium alone had been employed. Moreover, most of the isolations on eosin-methylene blue agar were obtained from patients early in the course of their disease, when many dysentery bacilli were being constantly excreted in the stools. After the diarrhea had subsided, however, and the pathogenic bacteria were excreted only intermittently and in small numbers, the value of the selective media became even more apparent. In 7 patients, from two to six weeks after symptoms had disappeared and the persons were apparently healthy, these media revealed 22 positive stools. The same stools were completely negative when cultured on eosin-methylene blue agar.



A comparison of the cultures on the two selective media shows that SS agar was somewhat more efficient than desoxycholate-citrate agar for the isolation of Flexner dysentery bacilli, although the number of specimens examined was relatively small, and no statistical evaluation of the media could be made. However, the SS agar was found to be superior to the desoxycholate-citrate agar in three noteworthy respects: (1) The inhibition of coliform bacteria was more marked; (2) the colonies of dysentery bacilli were more clearly defined; (3) the colonies of dysentery bacilli were usually more numerous.

Our findings, therefore, confirm those of Mayfield and Gober,<sup>3</sup> and indicate that SS agar is a valuable selective medium for the isolation of Flexner dysentery bacilli from the feces.\* We feel that the use of SS agar will increase the efficiency of methods for the bacteriologic examination of the feces, a matter of importance to the public health from the standpoint not only of diagnosing enteric disease, but also of recognizing carriers of the intestinal pathogens.

### CONCLUSIONS

SS agar is as efficient as desoxycholate-citrate agar for the isolation of Flexner dysentery bacilli.

The medium is superior to desoxycholate-citrate agar as regards inhibition of coliform bacteria, colony differentiation, and growth-promoting qualities for the pathogens.

The combination of SS agar and a suitable nonselective medium furnishes a reliable method for the isolation of the intestinal pathogens. For maximal efficiency two or more plates of the selective medium should be employed.

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\*We have also used SS agar to culture a series of stools from two patients with typhoid fever, one with Sonne dysentery, and one with enteritis caused by *Salmonella panama*. The results indicated that the medium was equally effective for the isolation of these organisms.

## A NOTE ON THE "BLACK LINE" IN HEMATOCRIT DETERMINATIONS\*

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IT MAY be a common observation that a narrow black band appears in hematocrit determinations at the top of the erythrocyte column, though the textbooks of hematology<sup>1-5</sup> and the literature<sup>7-9</sup> here listed make no reference to it. Neither Hedin in his pioneer work on the hematocrit nor Koeppé, who first described the development of translucence of erythrocytes when blood is centrifuged in hematocrit tubes at high speed, makes mention of the black line. It is, therefore, considered worth while to mention this phenomenon and to describe some simple experiments which establish its nature.

Oxalated human blood drawn directly from the cubital vein is introduced into Wintrobe hematocrit tubes capped with rubber, and centrifuged at about 2,500 r.p.m. for ten minutes. The tubes then show a lower column of erythrocytes and an upper column of plasma with about one division (1 per cent) of leucocytes above the erythrocytes. Immediately below the leucocytes a dark line is present, varying in intensity and width, but usually equal to about 0.5 per cent by volume. When hemolysis is great, black lines are seen both above and below the leucocytes.

The plasma and leucocytes may be pipetted off without disturbing the black layer, but it disappears on standing exposed to air, becoming red. If saline solution is now added to the 100 per cent mark, and the erythrocytes are resuspended and then centrifuged down, no dark line appears, obviously because there are no leucocytes to consume the oxygen. However, if a few yeast cells, *S. cerevisiae*, are now added to the top of the saline, in the above experiment, and are then centrifuged down, so that they rest on the top of the erythrocyte column, the dark band reappears as a result of oxygen consumption by the yeast cells. If blood is chilled and, while ice cold, is introduced into the hematocrit, on centrifugation, no black line appears at first but develops after a few minutes' standing at room temperature, when it increases in width gradually until a maximum width is reached. If the temperature is now increased to 37° C., the black line again increases in width with time, finally reaching a new maximum width of not more than 1 per cent. The width of the line is a function of the rate of oxygen consumption by the leucocytes as influenced by temperature.

Evidently the black line is a layer of erythrocytes in which the oxyhemoglobin has been reduced to hemoglobin by the metabolic activity of the leucocytes.

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The black line is thus reversible and can be produced by any agency using up oxygen locally at a rate greater than the diffusion rate of oxygen in the plasma or from distant erythrocytes. Perhaps the rate of increase in width of the line may be a useful indicator. For instance, if the width of the line is unequal in two samples of blood, while the leucocytic count is equal, one would expect a qualitative difference in the leucocytes.

I wish to acknowledge the helpful advice of Dr. Carl Moore, Washington University Medical School, and the kindness of Dr. W. C. Herold, St. Louis, in furnishing blood samples.

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## CHEMICAL

### A MICROBIOLOGICAL ASSAY METHOD FOR SIX B VITAMINS USING LACTOBACILLUS CASEI AND A MEDIUM OF ESSENTIALLY KNOWN COMPOSITION\*

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IT IS generally agreed that there is a great need in current vitamin research for rapid methods for the quantitative determination of vitamins. This is particularly true in the case of the vitamins of the B complex.

Since many B complex vitamins required by animals are essential in bacterial metabolism, microbiological assays, i.e., vitamin assays employing microorganisms as test agents, have rapidly gained approval. As our knowledge of bacterial nutrition has accumulated, these procedures have increased in number and have been revised and amplified. Microbiological assays are not only extremely sensitive to minute amounts of test material, but are accurate as well, and have the additional advantage over animal assays in being rapid and inexpensive. Furthermore, in the case of folic acid, microbiological technique is the only known method of assay.

Microbiological procedures in current use include assays for riboflavin (*Lactobacillus casei*),<sup>1</sup> pantothenic acid (*L. casei*),<sup>2</sup> pyridoxine (*Saccharomyces cerevisiae*),<sup>3</sup> nicotinic acid (*L. arabinosus*),<sup>4</sup> folic acid (*Streptococcus lactis*),<sup>5</sup> and biotin (*S. cerevisiae*, *Clostridium butylicum*).<sup>6, 7</sup> During the preparation of this manuscript a paper by Shull, Hutchings, and Peterson<sup>8</sup> describing a microbiological assay for biotin utilizing *L. casei* appeared. From the foregoing examples it is obvious that microbiological assays of the various B vitamins have necessitated use of a variety of organisms, culture media, and test conditions. Although the majority of these assays has been satisfactory, it would obviously be advantageous to have a single acidimetric method for members of the B complex, using only one test organism and a simplified basal medium of known composition. The organism presenting the greatest possibilities as an assay agent under these conditions is *L. casei* because (1) its nutritional requirements have been extensively investigated and have been found to include a majority of the B complex vitamins; (2) its acid production, which is easily and accurately determined, is a function of its growth; and (3) many laboratories are already familiar with its use in riboflavin and pantothenic acid assays.

At the time this study was begun, the status of the nutrition of *L. casei* was as follows: It had been demonstrated that this organism required ribo-

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†American type culture collection No. 7469.

flavin,<sup>1</sup> pantothenic acid,<sup>10</sup> nicotinic acid,<sup>10</sup> and pyridoxine.<sup>11</sup> The essential growth factors of unknown composition, other than those possibly present in hydrolyzed casein, were supplied by yeast extract and could be separated by adsorption on norit A into "filtrate" and "eluate" fractions.<sup>11</sup> Evidence had been presented indicating that the activity of the eluate fraction was due to "folic acid."<sup>5, 12</sup> However, the composition of the filtrate fraction was still unknown.

We, therefore, undertook (1) replacement of the yeast filtrate fraction with known substances which would permit maximum growth of *L. casei* in a medium of essentially known composition, and (2) thus, having determined the nutritional requirements of *L. casei*, to use this information in establishing assay procedures for the various vitamins this organism requires.

#### EXPERIMENTAL

At the time our studies on the nutrition of *L. casei* were begun, an investigation was in progress in this laboratory on the use of avidin in determining the biotin requirement of microorganisms.<sup>13</sup> A correlation had been established between growth inhibition by avidin and the biotin requirement of an organism. In general, those organisms which have been found to require biotin supplied in the culture medium were inhibited by avidin, while those organisms which were found to synthesize biotin<sup>14</sup> were not affected. The growth and acid production of *L. casei* were completely inhibited by avidin, and this inhibition could be readily reversed by the subsequent addition of crystalline biotin. This was taken as evidence that *L. casei* requires biotin as a growth essential.

Employing the basal medium of Snell and Peterson<sup>11</sup> containing pyridoxine and filtrate fraction of yeast extract as prepared by them, and with their norit eluate fraction replaced by "folic acid" in excess, we found that biotin would replace the filtrate fraction, yielding equivalent growth. Since we have not always been able to obtain maximum growth with the filtrate fraction, it was suspected that this constituent might contain insufficient biotin to support maximum growth. Biotin assay<sup>6</sup> of the filtrate fraction revealed that when prepared and used according to Snell and Peterson, a culture would be supplied with 0.0001 $\gamma$  of biotin. This amount was subsequently found insufficient for maximum growth of *L. casei*.<sup>5</sup> Peterson has recently stated, "the filtrate fraction can be replaced by biotin, thus leaving the eluate fraction as unknown."<sup>12</sup> However, no evidence was offered to support this statement.

In an effort to obtain maximum growth consistently the effect of a variety of growth accessory substances was investigated. As a result of the findings of Snell and Mitchell<sup>15</sup> that purine and pyrimidine bases are growth substances for lactic acid bacteria, adenine, guanine, xanthine, and uracil were included in the medium. These bases, together with biotin and folic acid concentrate, consistently supported maximum growth. We later added asparagine, since Feeney and Strong<sup>16</sup> had found it to be a growth accelerator, particularly

\*From our tests with Snell and Peterson's filtrate fraction, and as a result of biotin assay of this material, it would appear likely that under their conditions of test, the filtrate fraction supplied only a portion of the total biotin necessary for maximum growth. The remainder of the biotin necessary for maximum growth was probably supplied by their norit eluate concentrates, since their method of preparation might also concentrate biotin.

during the first twenty-four hours of incubation. The vitamins and growth accessories, as given in Table I, completely replace yeast extract, which is considered<sup>11</sup> the best source of growth essentials for supporting maximum growth and acid production of *L. casei*.

TABLE I  
COMPLETE MEDIUM FOR LACTOBACILLUS CASEI

|  |                      |
|--|----------------------|
| Casein hydrolysate   | 0.5 Gm. (dry weight) |
| Sodium acetate ( $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3 \text{H}_2\text{O}$ ) | 0.6 Gm.              |
| Glucose  | 1.0 Gm.              |
| Asparagine   | 25 mg.               |
| Tryptophane  | 10 mg.               |
| Cystine  | 10 mg.               |
| Salt solution A  | 0.5 c.c.             |
| Salt solution B  | 0.5 c.c.             |
| Guanine hydrochloride  | 500 $\mu\text{g}$    |
| Adenine sulfate  | 500 $\mu\text{g}$    |
| Xanthine   | 500 $\mu\text{g}$    |
| Uracil   | 500 $\mu\text{g}$    |
| Thiamine hydrochloride   | 10 $\mu\text{g}$     |
| Biotin (free acid)   | 0.5 $\mu\text{g}$    |
| Folic acid concentrate (50 per cent folic acid)*                                 | 1.0 $\mu\text{g}$    |
| Riboflavin   | 20 $\mu\text{g}$     |
| Calcium pantothenate   | 20 $\mu\text{g}$     |
| Nicotinic acid   | 20 $\mu\text{g}$     |
| Pyridoxine hydrochloride   | 40 $\mu\text{g}$     |
| Distilled water to   | 100 c.c.             |

pH adjusted to 6.8

|                          |         |  |           |
|--------------------------|---------|--|-----------|
| Salt solution A          |         | Salt solution B                            |           |
| $\text{K}_2\text{HPO}_4$ | 5 Gm.   | $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ | 10 Gm.    |
| $\text{KH}_2\text{PO}_4$ | 5 Gm.   | $\text{NaCl}$                              | 0.5 Gm.   |
| Water                    | 50 c.c. | $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ | 0.5 Gm.   |
|                          |         | $\text{MnSO}_4 \cdot 2 \text{H}_2\text{O}$ | 0.337 Gm. |
|                          |         | Water                                      | 250 c.c.  |

\*Kindly supplied by Dr. R. J. Williams, Department of Chemistry, University of Texas, Austin, Texas.

**Medium.**—The composition of our complete basal medium is given in Table I. It is essential that all constituents be of the highest purity. Best results are obtained if the medium is prepared just before use. Stock solutions of tryptophane, cystine hydrochloride, salt solutions A and B, and purine bases may be kept on hand, using toluene as a preservative where necessary. Since the purine and pyrimidine bases are not readily soluble in water, it has been found convenient to dissolve them in the casein hydrolysate. After the addition of riboflavin to the medium, exposure to light should be avoided.

It is necessary that great care be taken in the preparation of the casein hydrolysate, since this is the only constituent whose composition is not chemically defined; it is, therefore, the only source of variation. Purified or "vitamin-free casein"\* should be reprecipitated several times in order to remove traces of interfering vitamins, particularly nicotinic acid and pyridoxine. Reprecipitation is followed by the usual acid hydrolysis, and the resulting hydrolysate is treated with activated carbon, primarily for the removal of any biotin present. Culture medium containing casein hydrolysate prepared in this fashion supports maximum growth of the test organism and yields satisfactory blanks for all the six vitamin assays.

\*Most caseins of this type are "vitamin free" to the extent of being satisfactory for animal assays, but they contain small quantities of vitamins sufficient to interfere with microbiological assays.

*Reprecipitation of Casein.*—To one liter of distilled water at 50° C. 100 Gm. casein are added gradually with stirring. This mixture is stirred for thirty minutes, 3 Gm. of sodium bicarbonate (dissolved in a convenient volume of water) are added, and the stirring is continued until the casein is dissolved. It may be necessary to add more water. When the solution is complete, the pH is adjusted to 4.6 with 10 per cent hydrochloric acid. The casein precipitate is allowed to settle and is removed by filtration. The reprecipitation procedure is repeated several times, depending upon the purity of the original casein. As a rule, several reprecipitations are necessary before satisfactory blanks can be obtained in culture medium prepared with the resulting hydrolysate.

*Preparation of Casein Hydrolysate.*—One hundred grams (dry weight) of the reprecipitated casein are hydrolyzed with 500 c.c. of 25 per cent sulfuric acid for ten hours at 15 pounds pressure. The sulfuric acid is neutralized with barium hydroxide and the barium sulfate is removed by filtration.

*Treatment of Casein Hydrolysate With Activated Carbon.*\*—Casein hydrolysate solution in a concentration of 10 per cent (total solids) is acidified to pH 3 and treated with Nuchar (1 Gm. of Nuchar to 10 Gm. of casein). The mixture is stirred for one hour and filtered through Filter-cel. The resulting hydrolysate may be preserved under toluene or sterilized by autoclaving.

*Assay Procedure.*—When we succeeded in obtaining maximum growth of *L. casei* in a medium of essentially known composition, we turned to the problem of establishing assay procedures. It was found that, for any one essential vitamin, growth and acid production were consistently proportional, within a limited range, to the amount of vitamin supplied. This quantitative response formed the basis for the following assay method:

For the assay of any one of the six vitamins, basal medium of twice the concentration given in Table I is prepared with the omission of the vitamin under test. Dilutions of the sample to be assayed are placed in culture tubes with sufficient distilled water to make a total volume of 5 c.c. in each tube. Five cubic centimeters of medium (twofold concentration) are added, giving a final total volume of 10 c.c. per tube. Similarly, dilutions of the vitamin used as a standard are prepared. The tubes are plugged with cotton and sterilized at 15 pounds pressure for fifteen minutes. After cooling, the tubes are inoculated with a suspension of *L. casei* and are incubated at 37.5° C. for seventy-two hours. Following incubation, the amount of growth is determined by titration of the acid produced, using 0.1 N sodium hydroxide. Titration may be colorimetric with bromthymol blue as the indicator, or electrometric, using either glass or quinhydrone electrode. Values obtained from the dilutions of the vitamin standard are used to construct a standard curve from which the vitamin content of any dilution of sample may be calculated. Typical standard curves for all six vitamins required by *L. casei* are presented in Fig. 1.

*Preparation of Inoculum.*—Stock cultures of *L. casei* are carried in yeast dextrose agar slabs and are transferred as recommended by Snell and Strong.<sup>1</sup> A twenty-four-hour culture of *L. casei* in 10 c.c. of yeast dextrose broth is centrifuged, and the bacteria are washed in 10 c.c. of 0.85 per cent sodium

\*Nuchar XXX, Industrial Chemical Sales Co., Inc., 230 Park Ave., New York, N. Y.

chloride solution. The washed sediment is resuspended in 4 c.c. of saline, and 1 c.c. of the resulting suspension is added to 85 c.c. of saline. One-tenth cubic centimeter of this dilute suspension per tube serves as the inoculum.

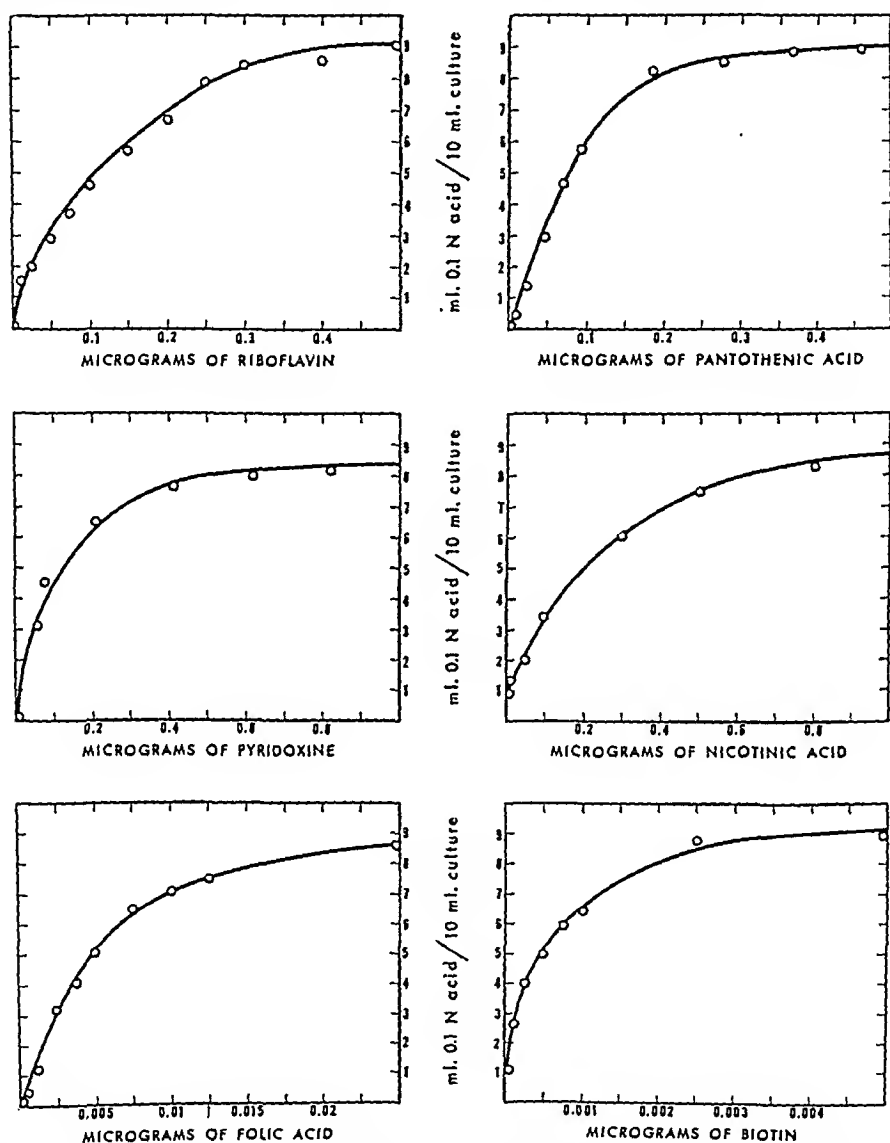


Fig. 1.—Standard assay curves for six B vitamins using *Lactobacillus casei* and a medium of essentially known composition.

#### DISCUSSION

From the data presented, it is clear that excellent growth of *L. casei* is obtained when riboflavin, pantothenic acid, nicotinic acid, folic acid, pyridoxine, and biotin are supplied in a suitable basal medium. Omission of any one of these essential vitamins will result in absence of growth and acid production. In the case of pantothenic acid, riboflavin, folic acid, and pyridoxine perfect blanks (no growth or acid production) were obtained. In the nicotinic acid



and biotin blanks a small amount of growth occurred, equivalent to approximately 1.0 c.c. of 0.10 N acid. Blanks of this magnitude are not prohibitive and do not interfere with accurate assay determinations. These blanks are due to traces of nicotinic acid and biotin which may ultimately be removed from the constituents of the medium.

For all six vitamins growth and acid production are, within a limited range, proportional to the concentration of the vitamin under test. The extreme upper and lower portions of the standard curve do not give reliable results, and only the portion of the curve which is linear, or approximately so, should be used for the calculation of assay values. When solutions or extracts of samples are run at several dilutions that fall within the accurate portion of the standard curve, values obtained at these different levels check closely.

The amount of each essential vitamin necessary for detectable growth of *L. casei* differs from one vitamin to another, but is minute in every case. This response to such small quantities provides sensitive tests for these vitamins. Because of this sensitivity, a sample to be assayed may be considerably diluted, thus eliminating or reducing the effect of nonspecific growth-stimulatory substances.

Preparation of the sample is an important part of the test, and one which, in the past, has not received the attention it merits. Liquid samples or water-soluble materials may be assayed without further treatment. Where the sample is particulate, autoclaving in large volumes of water at acid pH will, as a rule, provide quantitative extracts of most of the B complex vitamins. However, where the vitamin exists in combined form, as in the case of biotin, it is necessary to hydrolyze the sample in order to release the vitamin for utilization by *L. casei*. The methods for preparation of samples and the importance of such procedures, especially for biotin assay, have recently been reviewed.<sup>7, 17-19</sup>

The vitamin content of various naturally occurring materials and concentrates was determined both by our assay procedure and by established microbiological methods. Such parallel comparison assays of yeast and rice bran concentrate for riboflavin by the Snell and Strong method and for pantothenic acid by the method of Pennington, Snell, and Williams were in excellent agreement, while results of our biotin assay of a biotin concentrate compared favorably with values obtained by the yeast growth method of Snell, Eakin, and Williams. Although comparison with other methods has not been made for nicotinic acid, folic acid, and pyridoxine, it is probable that satisfactory results would be obtained, since the response of *L. casei* to these vitamins is similar in sensitivity, magnitude of blank, and type of standard curve to that obtained with riboflavin, pantothenic acid, and biotin.

#### SUMMARY

1. Maximum growth and acid production of *L. casei* have been obtained in a medium in which all constituents, except casein hydrolysate, are chemically defined.

2. Using this medium of essentially known composition with *L. casei* as the assay agent, it has been possible to establish a general assay procedure

for the vitamins essential for growth and acid production by this organism, viz., pantothenic acid, riboflavin, nicotinic acid, pyridoxine, folic acid, and biotin.

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## ON THE DETERMINATION OF URINARY CHOLESTEROL\*

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IN 1933, Mirsky<sup>1</sup> described a colorimetric procedure for the estimation of urinary cholesterol based upon the observation of de Toni,<sup>2</sup> who found that the tungstic acid precipitate of blood contained all the cholesterol. The method proposed by Mirsky utilized the addition of a 50 per cent solution of sodium tungstate to urine, and the subsequent slow addition of concentrated sulfuric acid to precipitate the tungstic acid. When observations on the excretion of cholesterol in the urine in patients with cancer were begun in this laboratory,<sup>3</sup> it was found that this procedure, at times, failed to recover satisfactory amounts of serum cholesterol added to the urine. It was assumed that urines, particularly from persons without renal disease, contained an insufficient amount of protein to carry down all the cholesterol. The addition of egg albumin to the urine was found to be a necessary step in order to obtain satisfactory recoveries of added cholesterol.

*Preparation of Egg Albumin Solution.*—The separation of the egg albumin from the yolk must be done carefully, since it is known that egg yolk contains appreciable quantities of cholesterol. The egg white was diluted with 9 volumes of distilled water and the solution was filtered through fluted filter paper. The cholesterol content of each batch of diluted egg albumin was determined; small amounts of cholesterol were invariably present, but the quantities in different batches were found to be remarkably constant (0.15 mg. in 100 c.c. of diluted egg albumin solution). Corrections for the cholesterol content of the added egg white were made in final calculations.

### METHOD

One hundred cubic centimeters of urine are placed in a 150 c.c. beaker or other suitable container. The urine is acidified with 0.5 c.c. of concentrated sulfuric acid, and 10 c.c. of the diluted egg albumin solution are added. After mixing, the albumin is precipitated by the addition of 5 c.c. of 10 per cent solution of sodium tungstate. The contents are again well mixed, and the precipitate is allowed to settle out for five to ten minutes. The solution is now filtered through a fluted filter paper (previously rendered fat free by washing in alcohol and ether). The protein precipitate is washed repeatedly with hot distilled water; the extent of the washing varies with the quantity of urinary pigment in the protein coagulum (50 c.c. to 100 c.c. of water usually suffice).

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The precipitate and filter paper are transferred to a 150 c.c. beaker, and 30 c.c. of a 3:1 alcohol-ether mixture are added. This is allowed to stand for fifteen to thirty minutes or, if more convenient, overnight. The beaker is then placed on an electric hot plate. The contents are brought to a boil and are then filtered through fat-free filter paper into another beaker of the same capacity.

TABLE I  
RECOVERY OF ADDED SERUM CHOLESTEROL

| NO. | URINE       |                   | DILUTED EGG ALBUMIN ADDED |                   | SERUM CHOLESTEROL ADDED (MG.) | URINARY CHOLESTEROL |             | GAIN OR LOSS (%) |
|-----|-------------|-------------------|---------------------------|-------------------|-------------------------------|---------------------|-------------|------------------|
|     | VOL. (C.C.) | CHOLESTEROL (MG.) | VOL. (C.C.)               | CHOLESTEROL (MG.) |                               | CALCULATED (MG.)    | FOUND (MG.) |                  |
| I   | 100         | 0.223             | 10                        | 0.015             | 0.133                         | 0.371               | 0.397       | +7               |
|     | 100         | 0.223             | 10                        | 0.015             | 0.266                         | 0.504               | 0.458       | -9               |
|     | 100         | 0.223             | 10                        | 0.015             | 0.398                         | 0.636               | 0.620       | -2               |
|     | 100         | 0.223             | 10                        | 0.015             | 0.531                         | 0.769               | 0.781       | +1               |
| II  | 100         | 0.128             | 10                        | 0.015             | 0.174                         | 0.317               | 0.298       | -6               |
| III | 100         | 0.171             | 15                        | 0.023             | 0.133                         | 0.327               | 0.324       | 0                |
|     | 100         | 0.171             | 15                        | 0.023             | 0.266                         | 0.460               | 0.463       | 0                |
|     | 100         | 0.171             | 15                        | 0.023             | 0.398                         | 0.592               | 0.551       | -6               |
|     | 100         | 0.171             | 15                        | 0.023             | 0.531                         | 0.725               | 0.688       | -5               |
| IV  | 100         | 0.141             | 10                        | 0.015             | 0.664                         | 0.820               | 0.806       | -2               |
|     | 100         | 0.141             | 10                        | 0.015             | 0.797                         | 0.953               | 0.993       | +4               |
| V   | 100         | 0.114             | 10                        | 0.015             | 0.930                         | 1.059               | 1.063       | 0                |
|     | 100         | 0.114             | 10                        | 0.015             | 1.063                         | 1.192               | 1.230       | +3               |
|     | 100         | 0.114             | 10                        | 0.015             | 1.195                         | 1.324               | 1.351       | +2               |

TABLE II  
DUPLICATE DETERMINATIONS

| VOL. OF URINE USED (C.C.) | TOTAL CHOLESTEROL (MG.) |       | TOTAL CHOLESTEROL (MG./100 C.C.) |       | MEAN DEVIATION (%) |
|---------------------------|-------------------------|-------|----------------------------------|-------|--------------------|
|                           | A                       | B     | A                                | B     |                    |
| 2 x 300                   | 0.670                   | 0.714 | 0.223                            | 0.238 | 5.3                |
| 2 x 300                   | 0.682                   | 0.688 | 0.227                            | 0.229 | 0.4                |
| 2 x 300                   | 0.701                   | 0.707 | 0.234                            | 0.236 | 0.4                |
| 2 x 300                   | 3.947                   | 3.750 | 1.316                            | 1.250 | 2.6                |
| 2 x 300                   | 0.444                   | 0.460 | 0.148                            | 0.153 | 1.6                |
| 2 x 300                   | 0.559                   | 0.547 | 0.186                            | 0.182 | 1.1                |
| 2 x 300                   | 0.556                   | 0.559 | 0.185                            | 0.186 | 0.3                |

Two additional extractions with alcohol-ether are made, and the filtrates are combined. The alcohol-ether extract is placed in an incubator (37° C.) and evaporated to dryness. To the dry residue in the beaker 30 c.c. of petroleum ether (B. P. 30° to 50° C.) are added. The beaker is placed on a hot plate and the petroleum ether is evaporated to approximately 10 c.c. volume. This is filtered through fat-free cotton or filter paper into another beaker; two additional petroleum ether extractions are carried out, and extracts are combined. This extract is placed in an incubator (37° C.) and is evaporated to dryness. The residue is extracted with 5 c.c. of chloroform, the chloroform extract is transferred to a graduated cylinder of 10 c.c. capacity; 2 c.c. of acetic anhydride and 0.1 c.c. of concentrated sulfuric acid are added. A standard solution containing 0.5 mg. of cholesterol in 5 c.c. of chloroform is prepared simultaneously for the development of the Liebermann-Burchard color reaction.<sup>4</sup> A red filter (Wratten 71-A) is used to facilitate color matching.

Calculation.—  $\frac{R_s}{R_u} \times 0.5 \times \frac{100}{X} = \text{mg. cholesterol in 100 c.c. of urine}$

Where  $R_s$  = reading of standard

$R_u$  = reading of unknown

$X$  = volume of urine extracted

For the determination of cholesterol in normal urine approximately 300 c.c. of urine must be extracted if the twenty-four-hour-urine volume does not exceed 1,500 c.c. With dilute urines, larger amounts must be extracted. This is done, preferably, by working with 100 c.c. quantities and combining the petroleum ether extracts.

#### RESULTS

Tables I and II are self-explanatory. The satisfactory recovery of added cholesterol to urine and the close correlation of duplicate determinations are shown in the protocols.

#### SUMMARY

A satisfactory colorimetric procedure for the determination of urinary cholesterol employing the addition of egg albumin to bind the cholesterol is described.

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## AN IMPROVED PYKNOMETER FOR BLOOD SERUM\*

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FOR the direct determination of the specific gravity of blood serum, as well as for the standardization and checking of the various indirect methods, a specially constructed specific gravity bottle, introduced by Moore and Van Slyke,<sup>1</sup> has been extensively used. However, precise adjustment of the volume of the liquid is generally troublesome. The uncertainty of seating of the ground stopper and the tendency of serum to seep up by capillary attraction between the stopper and the neck cause significant errors unless time-consuming measures are taken for their prevention. These difficulties are not encountered in a pycnometer of the pipette type, such as the one employed by Brown and Clark,<sup>2</sup> but it is always necessary to adjust the meniscus in the capillary exactly to a calibration mark. The pycnometer here described provides a more rapid and precise measurement by utilizing the principle of automatic volume adjustment employed in the well-known Tremmer blood-diluting pipette.

This pycnometer, which is not difficult to construct, consists of a thin-walled bulb *D* sealed to bent capillary tubes *C* and *E*, as shown in Fig. 1. The internal diameter of *E* is about 0.5 mm., and that of *C* is somewhat greater, but the tip *A* is drawn down to approximately the same size as *E*. The end of the capillary at *H* is a ground and polished surface at right angles to the longitudinal axis, and represents the point to which the liquid is brought in filling the device. A small bulb *F* serves to prevent the liquid from entering the capillary *G* too rapidly after filling the main bulb.

Sizes from 1 to 2 ml. have been found to be satisfactory. If many samples are to be measured, a considerable saving in time may be effected by constructing the pycnometer to contain an integral number of grams of water at 20° C. This is accomplished conveniently by expanding or shrinking the bulb before bending the capillary stems. Subsequent bending of the tubes produces a slight decrease in volume, permitting final adjustment to be made by enlarging the capillary at *B* and grinding the tip.

In using the pycnometer, a rubber suction tube is attached to the end *I*, and the serum contained in the short test tube *T* is drawn up to the small bulb *B*. The test tube is then tilted at an angle while the end *E* is lowered, permitting the liquid to flow to *H* by gravity and capillary action. The pycnometer is next removed from the test tube and quickly wiped with a damp cloth, while *A* is maintained at a slightly higher level than *H* in order to lessen the chance of drawing the liquid away from the latter.

Measurements are made at room temperature and the weight at 20° C. is estimated by applying a suitable correction. The number of milligrams to be

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added to, or subtracted from, the weight at room temperature to give the weight at 20° C. may be obtained from a chart prepared by Moore and Van Slyke.<sup>1, 2</sup> In order to avoid warming the liquid, the pyknometer is filled as quickly as possible, care being taken to handle it only by the stems at points well removed from the bulb. Loss of liquid due to expansion during weighing may be prevented by forcing a small drop out of the capillary at *H*. With these precautions satisfactory results can be obtained without the use of a thermostat. Evaporation of the serum will ordinarily cause no significant decrease in weight. In one test the loss was less than 0.1 mg. when the filled pyknometer was left in the balance case for one-half hour.

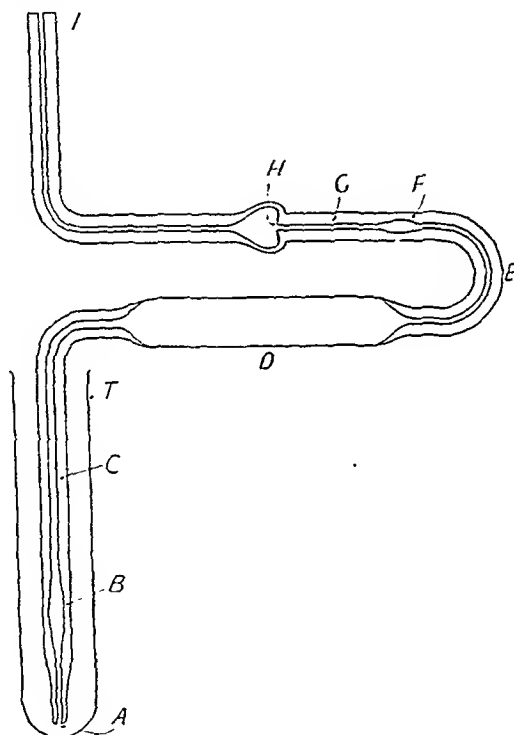


Fig. 1.—Pipette pyknometer with automatic volume adjustment.

The pyknometer is capable of yielding results which are accurate within one or two units in the fourth decimal place, with a minimum expenditure of time and effort. In a long series of measurements on the same sample, using the 1 ml. size which was cleaned, dried, and refilled for each determination, the maximum deviation from the mean specific gravity was less than  $2 \times 10^{-4}$ .

Although designed for use with blood serum, the device obviously may be employed for determining the specific gravity of other aqueous solutions.

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# MEDICAL ILLUSTRATION

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## WAX-RESIN COMPOSITIONS FOR MOULAGE-MAKING

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IN THE April, 1942, issue of this JOURNAL<sup>1</sup> I, together with a co-worker, described a basic agar composition for making molds or negatives of living and dead tissue to be used in moulage work. Since a cast or a positive is made in such a mold, I wish to confine the present article to the basic materials used in making the positive. There are numerous materials that can be employed for this purpose, such as wax-resin mixtures, celluloid, rubber, and a synthetic fleshlike gelatin mixture. Rubber has been discussed in a previous article.<sup>2</sup> Celluloid, as well as wax-resin mixtures, has been discussed to some extent in my book *Molding and Casting*.<sup>3</sup> Because of the shortage of rubber, experiments are in progress at the present time to develop a suitable substitute for making both positive and negative moulages. This work will be published at a later date.

The average novice has had greater success in working with waxes and resins for the positive; therefore, these materials will be considered here in some detail. Furthermore, the wax-resin mixtures lend themselves more readily to corrections. They also can be used over again in the event of a failure. When the moulage worker has become thoroughly familiar with rubber, celluloid, and gelatin mixtures, he will find that they are as simple to compound and use as the wax-resin mixtures. All these materials, when properly used, possess the translucency of flesh, which is the first essential in choosing any positive composition. In contrast to these materials, plaster of Paris and papier-mâché, which are among the most common positive materials, lack this translucency, and, for this reason, cannot be used with the same degree of success in making facsimile reproductions of protoplasmic objects.

Probably the greatest drawback to the use of wax-resin mixtures is the fact that the resulting cast is broken easily. This disadvantage is overcome when the cast is made of celluloid, rubber, or gelatin mixtures. However, the wax-resin cast is important because it is often used as an intermediate step in making casts of other materials. For example, in a rubber facial prosthesis a wax cast generally is made first and fitted to the patient before the rubber one is made. Necessary changes to obtain perfect fitting are easier to effect in wax than in rubber. Accordingly, a basic wax-resin formula will be considered in detail.

The wax-resin composition used for the positive impression should possess the following qualities:

1. It should be possible to brush or "paint" the positive composition into a plaster or agar mold without having the cast crack or lift from the surface of the mold.



2. The melting point should be so high that it will not be affected by natural heat in the hottest weather. A cast, no matter how thin, should never droop as a candle often does under normal summer temperatures.

3. It should not crack or shrink to any great extent when subjected to the normal cold of winter.

4. It should not crack under sudden changes of temperature, as does a thin layer of hot wax of a high melting point when poured into a cold mold.

5. The shrinkage of the positive wax should be so slight as to cause no cracks when it is shrinking against a rigid mold or after it has been mounted.

6. It should become set, yet remain pliable, at a degree of heat low enough for it to be bent or formed with the hands without breaking or cracking the wax or fear of burning the hands; nor should the composition stick to the hands. This pliability is advantageous, since it often becomes necessary to bend a positive composition into a more desirable shape. For example, it may be necessary to bend the positive to relieve it from some undercut surface on a rigid plaster mold. After the positive has been relieved of such an undercut, it can be rebent to its normal position.

These rigid requirements should be kept in mind when considering the published material on positive formulas.

#### ADVANTAGES OF BEING ABLE TO APPLY THE POSITIVE MATERIAL WITH A BRUSH

A positive wax-resin material that can be brushed, spread, or painted, instead of being poured into the mold, offers decided advantages which may be enumerated as follows:

1. It eliminates the danger of the much dreaded border lines, i.e., lines caused by the junction of hot and cold waxes on a smooth surface. The hot and cold waxes do not blend together, and as a result a line is formed where the waxes of different temperatures meet.

2. The application of the positive with a brush permits a freedom of working without the necessity of lifting heavy molds to turn them in various positions so that the wax will flow evenly and in the same thickness over the entire mold. This same method, when applied to large forms, will prevent a great outlay of physical strength and nerve-racking exertion, which also may require the assistance of helpmates to produce the cast.

3. It is far more economical to "paint" a mold than to pour one, because a quantity of wax may be lost or damaged beyond further use by dripping over the edges of the mold while the latter is being rotated, at the same time soiling one's clothing and burning the hands. The composition may fall on the floor, worktable, or any place except where it should go.

4. "Painting in" makes it possible to fill the separate sections of a piece mold before they are put in place and insures the surfaces of being well and evenly covered. After they have been put in their proper places, the positive pieces may be cemented together by the application of more wax, which may be poured or painted into the cracks between the positive pieces.

5. In painting with the positive substance rather than pouring it into the mold, it is possible to apply masses of different materials and colors next to

one another in the same layer. By the application of one translucent color over another, interesting and lifelike effects may be obtained. One may spread on the mold a wax composition to resemble the skin in color and texture to the edges of a gapping, moist, reddish wound, which would ordinarily glisten with high lights. This wound area of the cast can be painted in with a different material and color from that of the skin. While this second material may have a wax base, it can be incorporated with other materials, such as various gums, to produce the desired glossy effect.



Fig. 1.—A wax-resin mouldage of a hand affected with smallpox. This cast was made in a one-piece agar mold. A formula for pouring was used.

6. One may prepare a palette from an ordinary muffin pan and fill each cup with positive wax material which has been mixed with a different color. The caster may then use this muffin pan as an artist does his palette in painting a canvas; the caster paints his mold instead. The thin applications of the various colors on this mold must be strengthened or backed up by a thicker layer of wax, then reinforced with gauze, cheesecloth, or burlap fibers or threads.

In this manner the small blue vessels, which lie beneath the transparent skin, can be incorporated into the cast in the most naturalistic manner. Another indication for the use of color in the same way is a typical hemorrhage under the skin. The fresh flowing blood possesses a different color from stag-

nant blood. Thus the vessels may be painted red on a thin coating of semi-transparent positive formula which has been applied to the mold. These vessel details are then backed with more composition and finally strengthened with gauze, cheesecloth, or burlap. When the cast is finally removed from the mold, the vessels appear to lie under the skin rather than painted on the surface, as is so often done by the caster. It is interesting to note that *red* vessels under an ivory colored skin appear *blue* when viewed through the skin. This may be tested by placing a piece of ivory-colored tracing paper over dark red marks drawn on white paper. These red marks will seem to be blue, provided the right shade of tracing paper and the proper shade of red are used.

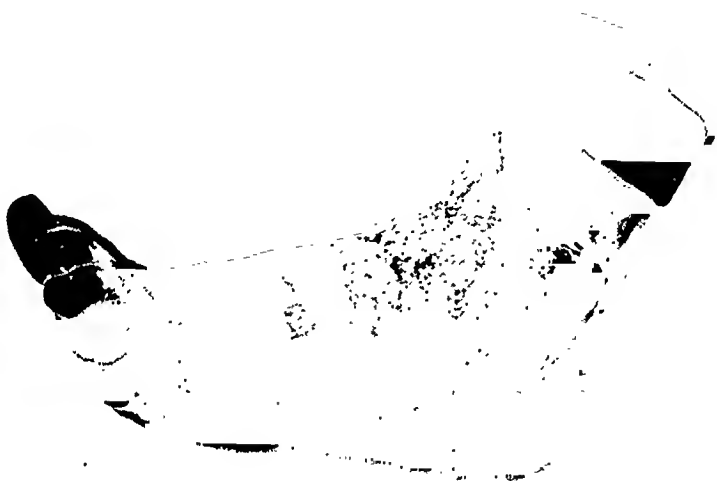


Fig. 2.—A wax-resin mouldage of a syphilitic gumma. This cast was made in a one-piece agar mold.

There are unlimited possibilities for the specific use of a positive material that can be painted into the mold rather than one that must be poured. In fact, these advantages are too numerous to mention. However, they will suggest themselves as the worker begins the making of molds and casts.

A simple basic formula for brushing into the mold is as follows:

|                          | PARTS BY WEIGHT |
|--------------------------|-----------------|
| Paraffin (about 55° C.)  | 8               |
| Carnauba wax             | 1               |
| Rosin, water-white, lump | 8               |
| Red oil-soluble dye      | q.s.            |

The following is a suitable formula for pouring into the mold:

|                          |      |
|--------------------------|------|
| Paraffin (about 55° C.)  | 8    |
| Carnauba wax (light)     | 1    |
| Rosin, water-white, lump | 2    |
| Red oil-soluble dye      | q.s. |

## RESIN\*

In spite of the fact that such work is often called "wax" casting, resin is the basic substance in those formulas which are the most simple to use and have the most versatility. Resin gives the compositions toughness and the quality of bending without breaking; it hardens more slowly, and prevents warping and cracking to a great extent. Common rosin or colophony is generally used for this purpose because of its cheapness. Bleached resin (i.e., water-white) serves better where the chromatic intensity is to be kept low. *The resin content should amount to about half of the mixture*, otherwise brushing of the positive mixture into the mold is sometimes rendered difficult and leaves "lap lines" or border lines. Such lines are formed where a hot mixture meets a cold or set mixture of the positive material and causes a line to appear at the junction of the hot and cold mixtures. Compositions containing a large percentage of resin do not flow easily into fine details unless they are painted into the mold.

If the rosin is powdered or if too much is applied, it may oxidize quite readily, turn dark, and separate from the mixture as a nonsoluble precipitate in the bottom of the pot (see Compounding).

## WAX

*Low Melting Point.*—The viscosity and viscidty, or internal friction, of resins are reduced by the addition of a wax. Such waxes are paraffin, white beeswax, ceresin, and spermaceti. Here again the cheaper product, paraffin, is generally chosen.

*High Melting Point.*—The melting point of a wax and resin composition may be raised by the addition of a wax having a high melting point. Such waxes are carnauba, montan, and Chinese wax. Carnauba, light, is usually selected. The amount of such a wax that is used in the composition should never be more than one-tenth the weight of the resin; otherwise shrinkage and brittleness of the entire composition will result.

In addition to raising the melting point of a wax-resin mixture, carnauba wax also improves the flowing qualities of the mixture and makes it possible to obtain a sharp impression of skin detail, such as the fingerprint pattern. If this wax is not included, the resulting cast may be soft and lack sharp, concise detail.

## COLOR

Where it is desirable to have the wax-resin mixture of a basic flesh tone which is composed of red and yellow, a permanent red oil-soluble dye is added. A yellow dye is not used because the yellow of the carnauba wax and resin is sufficient. The red dye mixes with the yellow of the wax and resin to produce the flesh tint. Of course, such a dye must be used sparingly. A gram or so of dye is dissolved first in about 200 c.c. of turpentine. An eye dropper is then

\*The term "resin" means an amorphous substance that exudes from plants. The term "rosin" indicates the residue remaining after the distillation of oil of turpentine. Generally, common rosin is the resin of the pine tree. It is also called colophony. Gum mastic, gum dammar, and similar substances are also resins but they are not rosins. Gum rosin darkens less rapidly than what is called wood rosin. Both are obtained from the pine tree. Gum mastic or gum dammar could be substituted for rosin (colophony) but these resins are decidedly more expensive.

used to place a drop at a time of this solution into the melted wax-resin mixture until the desired shade is reached. From time to time the resulting color may be observed by placing a few drops of the melted mixture on a piece of white paper. When this sets, it is carefully studied in daylight to see whether it is of the basic shade desired. The final monotone wax-resin cast is fixed with shellac or lacquer and then tinted with the proper oil-soluble pigments.

#### COMPOUNDING

Water is placed in the bottom section of a double boiler and brought to a boil. The fire is then cut down until the water just boils. Rapid boiling may cause the mixture to become too dark. This is important, because excess heat or prolonged heating causes the resin of the mixture to darken and separate as an insoluble resin. The waxes only are placed in the top section and allowed to melt completely before the resin is added. At no time must the pot be allowed to boil vigorously while the resin is mixed with the wax. Heating may be continued with occasional stirring, until the resin is melted completely. Prolonged heating causes it to darken. In fact, some workers prefer to melt the waxes thoroughly and then cut off all heat before adding the resin, depending on the stored-up heat in the waxes and the water section of the double boiler to melt the resin. This does not always suffice, but it will serve to impress upon the worker the importance of not getting the resin too hot.

The basic formulas herewith presented may be varied to suit specific purposes or they may be used as specified. Other waxes and resins may be substituted. Those given were chosen because their cost was decidedly less than waxes and resins having similar qualities, such as melting points and viscosity.

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# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**CIRRHOSIS, Hepatic, Serum Proteins in,** Post, J., and Patek, A. J. *Arch. Int. Med.* 69: 67, 1942; *Ibid.* 69: 83, 1942.

Of 61 patients with cirrhosis of the liver, 54 had an abnormal albumin-globulin ratio on admission to the hospital. These data confirm the reports of other authors.

The prognosis as to duration of life becomes increasingly grave as the level of serum albumin decreases. The levels of the serum globulin and the serum total protein have no such prognostic significance.

There is a direct correlation between the level of the serum albumin and the clinical course. Clinical improvement is associated with a rise in serum albumin toward normal. In instances of clinical failure there is no sustained rise.

The level of serum albumin is significantly lower in patients with ascites than in those without ascites.

Diuresis is associated with a rise in serum albumin. The mean value for serum albumin at which diuresis occurs is 3.1 Gm. per 100 c.c. (standard deviation, 0.2 Gm.).

From the data presented, it appears that a reduction in serum albumin is an essential factor in the formation of ascites.

Nitrogen balance studies were made on 5 patients with cirrhosis of the liver, ascites, and reduced serum albumin. Although the patients remained in positive nitrogen balance during the periods of high protein feeding, there was no correlated rise in the level of the serum albumin. In this respect these patients differ from persons with simple protein starvation.

The data recorded here indicate that patients with cirrhosis of the liver absorb and retain food protein. The evidence suggests that the mechanism for the synthesis of serum albumin is impaired.

**VAGINAL SMEARS, New and Rapid Method of Staining Based Upon a Specific Color Reaction for Glycogen,** Mack, H. C. *Harper Hosp. Bull.* 1: 54, 1942.

1. *Preparation of Smears.*—A moistened cotton applicator is inserted into the vagina and twirled slightly (one complete rotation) against the vaginal wall. The cotton end of the applicator is then rolled lengthwise over the surface of a clean glass slide. By rolling, rather than rubbing, a uniformly thin film of cells, with minimal clumping and cell distortion, results. The film dries almost immediately and may be stained at once.

2. *Staining of Smears.*—Staining is accomplished simply by laying the slide, face down, over a shallow dish containing a small amount of Lugol's solution. Iodine vapors which arise insensibly from the solution suffice to stain the glycogen-containing cells in two or three minutes. Microscopic examination may be carried out immediately. Although such stains fade in from twenty-four to forty-eight hours, restaining (by the same method) may be carried out repeatedly if later examinations are desired.

**SYPHILIS, Biologic False Positive Serologic Reactions in Tests for,** Mohr, C. F., Moore, J. E., and Eagle, H. *Arch. Int. Med.* 68: 1161, 1941.

The authors have reported the cases of 11 nonsyphilitic patients with various diseases in whom transitory biologic false positive serologic reactions for syphilis were observed.

When analyzed quantitatively, a low titer was usually revealed in these tests, but in the tests on 2 patients, one with a sore throat and one with pneumonia, a high titer, comparable to that seen in the early stages of syphilis, was observed.

It is suggested that a complement fixation test with spirochetal antigen (pallida test) may be helpful in differentiating false from true positive reactions.

In view of the enormous increase in the routine use of serologic tests for syphilis, the necessity for caution in their interpretation is emphasized.

**NOSE, Normal and Abnormal Bacterial Flora of, Jacobson, L. O., and Dick, G. F. J. A. M. A. 117: 2222, 1941.**

Cultures of nasal secretion were made as a matter of routine for 500 patients admitted to a general medical service, none of whom had been admitted because of a primary complaint referable to the nose or the paranasal sinuses.

The authors find in agreement with most observers that the normal nasal flora consists chiefly of *Staph. albus* and diphtheroid bacilli, and less frequently, of *Staph. aureus* and *M. catarrhalis*.

Green-forming streptococci and pneumococci are occasional transients in the nasal cavity.

The presence of streptococci, pneumococci, *B. mucosus*, Pfeiffer's bacilli, and diphtheria bacilli indicates disease of the nasal mucosa or sinus disease, or both.

The frequency with which green-forming streptococci, hemolytic streptococci, and pneumococci were isolated by culture of nasal secretion in cases of sinusitis, acute or chronic, shows the diagnostic value of this simple, inexpensive procedure.

**ANEMIA, The Significance of Target Cells in, Bohrod, M. G. Am. J. M. Sc. 202: 869, 1941.**

Target cells are commonly seen in the regeneration of blood, regardless of the cause of the blood loss.

In acute anemias they are present for only a short time early in the regeneration phase, and they have disappeared by the time a significant rise of erythrocyte count is evident. In chronic anemias they may be present over long periods of time.

The question of the normal shape of the circulating erythrocyte should be re-examined. It is possible, but not yet proved, that the target cell preserves the bowl shape of the intravascular cell in modified form and resists deforming influences which are responsible for producing the biconcave disc seen in blood outside the body.

The target cell is a hyperresistant cell produced by the bone marrow in response to the blood loss. Increased resistance to the hemolytic action of hypotonic saline and to acetic acid has been demonstrated.

The contention that the target cell represents the fundamental defect in Cooley's anemia seems to be unjustified. It is simpler, in the light of available evidence, to regard the target cell in this disease, as in sickle-cell anemia and many other anemias, as a response to the blood destruction rather than the cause of it.

**SYPHILIS, The Kahn Verification Test, Chargin, L., and Rein, C. R. Arch. Dermat. & Syph. 44: 1031, 1941.**

The reactions to the Kahn verification test were studied for 1,565 persons with various conditions, comprising syphilis, various nonsyphilitic dermatoses, questionable conditions, pregnancy, contagious diseases, pinta, malaria, leprosy, and miscellaneous conditions.

In the group of 349 syphilitic patients, who had received varying amounts of treatment, the verification tests gave the syphilitic type reaction in 100 per cent of those with strongly positive serodiagnostic reactions, in 76.5 per cent of those with weakly positive reactions, and in 40.2 per cent of those showing doubtful reactions. There were no reactions of the syphilitic type in the patients with negative serodiagnostic reactions. The incidence of the general biologic type of verification reaction in the group with negative serologic reactions was 7.1 per cent, and in the group with doubtful serologic reactions it was 12.1 per cent.

In the nonsyphilitic group, 269 in number, there were only 2 patients who had a syphilitic type of verification reaction. Syphilis could not be established in these patients. The incidence of biologic reactions in this group varied from 13.8 to 47.6 per cent.

In the questionable group of 253 members, 83 patients (32.7 per cent) gave a syphilitic type of verification reaction. Further study is necessary to determine whether they actually had syphilis. The incidence of the general biologic (nonsyphilitic) type of reaction in this group varied between 2.3 and 38.6 per cent.

In the group of patients with acute contagious diseases the general biologic (nonsyphilitic) type of verification reaction was obtained in between 29.3 and 64.0 per cent, and in the pregnant women this type reaction was obtained in between 21.2 and 55.5 per cent.

The highest incidence of the general biologic (nonsyphilitic) type of reaction is found to be associated with the doubtful serodiagnostic reactions in all groups.

The percentage of the syphilitic type of verification reaction increases with the increase in the titer of serodiagnostic reaction.

In the group of 268 patients with pinta, all of whom gave strongly positive serodiagnostic reactions, 83.9 per cent gave a syphilitic type of reaction to a verification test.

Agreements as well as discrepancies were observed in a number of blood specimens that were subjected to repeated verification tests.

**ZIEHL-NEELSEN STAIN, Sulphonamides and the, Mary John, Sr. Am. J. M. Technol. 7: 256, 1941.**

Observations are reported showing that after the administration of sulfonamide drugs the sputum may contain crystals which are acid-fast and which may rather closely resemble *B. tuberculosis*.

These are seen as long slender, acid-fast rods, sometimes curved, sometimes short and straight, sometimes in bristling clumps.

It is advisable that these drugs be discontinued before sputum examinations for tubercle bacilli are reported as positive.

**TRICHINOSIS, A New Precipitin Test for, Roth, H. Acta Path. et microbiol. Scandinav. 18: 160, 1941.**

Living infective larvae of *Trichinella spiralis*, when incubated at 37° C. in the sera of patients suffering from trichinosis or of guinea pigs or rabbits, experimentally trichinized, give rise to the formation in the sera of microscopically visible precipitates. However, no specific deleterious effect of such immune sera on the larvae has as yet been seen. The diagnostic value of this new microscopic test is compared with that of a simplified modification of the usual macroscopic Buchman precipitin test (using a filtrate of the saline extraction of dried trichina larvae as antigen). Both reactions seem to depend on the same antibodies present in immune serum, but the microscopic test may sometimes be more delicate in the demonstration of very slightly developed antibodies.

**SPUTUM, Physical and Chemical Properties of, Basch, F. P., Holinger, P., and Poncher, H. G.: Am. J. Dis. Child. 62: 981, 1941.**

The tracheobronchial tree of patients with bronchiectasis contains three essentially different types of sputum, which are not to be confused with the three layers into which the sputum of patients with bronchiectasis or abscess of the lung divides on standing.

To effect the series of the changes which occur spontaneously in the sputum during its movement from the lowest to the highest levels of the tracheobronchial tree, from which it may be coughed out, certain physical, chemical, and bacteriologic actions take place. The changes are not due to physical processes which cause liquefaction or thickening, because a parallel has not been found between the viscosity and the content of solid substances of the sputum.

The secretion from the most dependent portion of the tracheobronchial tree, designated as the third portion, is never coughed up, but is obtainable only by bronchoscopic suction. It has an extremely high viscosity and a high content of organic and inorganic substances. This sputum becomes even thicker after it is removed from the bronchi.



The third portion is moved to a higher level both by the tussive squeeze and by the pressure of secretions to form the second portion, which is found in the trachea and the larger bronchi. The second portion is a liquefied sputum which has a lower viscosity, a lower dried residue and a lower percentage ash than the third portion.

On the top of this secretion, after a rest period, a plug is found which has a moderately high viscosity but a dried residue and percentage of ash which are even lower than those of the second portion.

The sputum of patients with massive collapse of the lung shows a decrease in viscosity during the course of the disease in spite of a marked increase in its content of organic and inorganic material. This spontaneous dilution is probably due to bacterial action.

The characteristic properties of the sputum of patients with bronchiectasis, and post-operative massive collapse of the lung may be due to a variety of factors which may be summarized as follows: (1) selective secretion, (2) selective resorption, (3) speed of movement of secretions from one level to another, (4) bacterial action, and (5) combinations of the foregoing factors.

#### VAGINAL FLORA in Children, Hardy, G. C. J. Dis. Child. 62: 939, 1941.

In a study in which a limited attempt was made to ascertain the etiologic agents of vaginal infection in girls, the only bacteria which could be incriminated were the gonococcus, the group A streptococcus and, possibly, the diplobacillus of Petit.

In the case of the last two organisms, there is bacteriologic evidence, clinical evidence, or both, of an interrelation between infections of the upper respiratory tract and those of the genital tract.

The streptococcus found simultaneously in the upper respiratory and the vaginal tract were types 11, 24, 25, and 28.

Further study of the diplobacillus, which, superficially at least, may be confused with the gonococcus, is indicated. Gonorrhea should not be diagnosed without complete cultural studies.

Among the patients studied, very few, apart from the premature group, could be considered normal. The large number of purulent secretions found in the children in the hospital wards suggests that such a reaction may be the concomitant of a variety of illnesses or constitutional states. Saprophytic organisms appear to grow in the vaginas of such patients with little restraint. For information concerning normal vaginal flora of childhood the authors recommend study of a group of healthy girls.

More anatomic and histologic studies should provide a better understanding of vaginal infections, so that rational treatment could be devised. An attempt should be made, also, to give adequate clinical attention to patients with such infections as early in the course of the illness as possible.

#### CARBON DISULFIDE POISONING, Neuropathologic Changes in Experimental, in Cats, Ferraro, A., Jervis, G. A., and Flieler, D. J. Arch. Path. 32: 723, 1941.

Five cats were exposed to varying doses of carbon disulfide. The most important changes in carbon disulfide poisoning consist in (a) diffuse vascular involvement of the productive type, i.e., proliferation of capillaries and hypertrophy of the walls of blood vessels, often leading to endarteritis; and (b) diffuse neurocellular changes, ranging from chromatolysis to severe degeneration, diffusely scattered all over the brain and cerebellum.

Both cortical and subcortical structures are involved. The lesions are particularly evident in the corpora quadrigemina, the cerebellar nuclei, and the vestibular nuclei. The region of the cerebellar nuclei constitutes an area where the change is definitely predominant, and in 4 of 5 cases the pathologic involvement leads to bilateral softening of this region. Next to the cerebellar nuclei, the vestibular area is the one most intensely involved. In an animal in which the exposure to the action of the gas was most prolonged, softening was also found in the lenticular nucleus and in the substantia nigra.

## REVIEWS

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Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

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### The Diseases of the Eye\*

SURELY there are few medical students who have graduated since 1900 who are not well acquainted with May's little textbook. May has always kept it small, although it has perforce become a little larger during the years. A book which is now in its seventeenth American edition, has been translated into nine foreign languages, and has had forty-four editions in foreign lands, scarcely requires review.

### Infant Nutrition†

THE late Professor Marriott's excellent book has been revised by Dr. P. C. Jeans of the University of Iowa. The work starts with the growth of the infant and child and its physiologic development, particularly as regards nutrition. Then follow sections on the various types of metabolism, carbohydrate, protein, mineral, water, fat, and vitamin. The major portion of the volume is given to gastrointestinal and nutritional disturbances of infancy and childhood, with rather general discussion of remedial measures. General principles are emphasized. There is no padding with sample diets, case histories, and the like.

### Diseases of the Digestive System‡

A ROUTINE book covering the field indicated by its title, *Diseases of the Digestive System* is written by fifty well-known contributors. It seems to cover the field adequately and should be of service to gastroenterologists and to men practicing more general medicine.

### The Art and Science of Nutrition§

VERY profusely and well illustrated, this volume is written primarily for students of dietetics. It discusses normal nutrition, food requirements under special conditions, dietary therapy, and the choice, preparation, and serving of foods. In the last section, about 140 pages, there is a profusion of tables, recipes, and diets which should be of help to those whose interest is in the scientific preparation of foods.

\*Manual of the Diseases of the Eye. For Students and General Practitioners. By Charles H. May, M.D., Consulting Ophthalmologist to Bellevue Hospitals, New York. Formerly Chief of Clinic and Instructor in Department of Columbia University, and Director of the Eye Service of New York. Revised with the assistance of Charles A. Perera, M.D., College of Physicians and Surgeons, Medical Department of Columbia University, New York. Assistant Attending Ophthalmologist, Presbyterian Hospital, New York. Cloth, ed. 17, 519 pages, 387 illustrations, including 32 plates and 93 colored figures. William Wood and Company, Baltimore, Md., 1941.

†Textbook of Infant Feeding for Students and Practitioners of Pediatrics. By McKim Marriott, B.S., M.D., Late Professor of Pediatrics, Washington University School of Medicine; Physician in Chief, St. Louis Children's Hospital, St. Louis. Revised by P. C. Jeans, A.B., M.D., Professor of Pediatrics, College of Medicine, State University of Iowa, Iowa City. Cloth, ed. 3, 475 pages. The C. V. Mosby Company, St. Louis, Mo., 1941.

‡Diseases of the Digestive System. Edited by Sidney A. Portis, B.S., M.D., F.A.C.P., Associate Professor of Clinical Medicine, Rush Medical College of the University of Chicago; Attending Physician, Michael Reese Hospital; Consulting Physician, Cook County Hospital, Chicago. Cloth, 932 pages, illustrated with 176 engravings, \$10.00. Lea & Febiger, Philadelphia, Pa., 1941.

§The Art and Science of Nutrition. A Textbook on the Nutrition. By Estelle E. Hawley, Ph.D., and Grace Carden, B.S., 1941. School of Medicine and Dentistry, Rochester, N. Y. Cloth, 619 pages, including 12 in color, \$3.50. The C. V. Mosby Company, St. Louis, Mo., 1941.

### University of California Hospital Formulary\*

A POCKET compendium of useful information for internes and fourth-year medical students which would be appropriate for any hospital or any school; this Formulary contains such subjects as prescription writing, materia medica, discussion of buffered and isotonic solutions, dosage tables for various drugs, endocrine products, vitamin preparations, etc. Its therapeutic index suggests the appropriate drug for various symptoms and pathologic states. There are sections on special diets, obstetrical data, laboratory methods, and emergency procedures.

### The American and His Food†

A DELIGHTFULLY written history of food habits in the United States from pioneer days, this book presents the difference between menus on the farms and in the cities in early days that is especially interesting. Today, however, the farmer lives better than the city dweller did a century and a half ago.

The improvements in diet which were facilitated by improved transportation facilities rather naturally parallel the advances in commerce in general. The last portion of the book deals with present-day nutrition and needs for the future.

### The March of Medicine‡

THIS is the sixth annual compilation of a series of lectures to the laity sponsored by the New York Academy of Medicine. The material covered may be inferred from the list of subjects and their authors: Humanism and Science by Alan Gregg; Paracelsus in the Light of Four Hundred Years by Henry E. Sigerist; Psychiatry and the Normal Life by William Healy; Philosophy as Therapy by Irvin Edman; The Promise of Endocrinology by Oscar Riddle; and What We Do Know About Cancer by Francis Carter Wood.

### Essentials of Electrocardiography§

A COMPREHENSIVE discussion of basic principles, technique, and interpretation in the performance of clinical electrocardiography; this book is an excellent guide for students and a reference manual for clinicians.

### Diagnostic Procedures and Reagents||

THIS is a reasonably small book detailing technique for the laboratory diagnosis and control of the communicable diseases, published by the American Public Health Association. It is primarily a book on laboratory methods rather than on control, and as such is authoritative. In addition to the bacterial contagious diseases it covers rickettsial infections, syphilis, rabies, and pathogenic fungi.

### The Principal Nervous Pathways¶

THE *Principal Nervous Pathways* is a splendidly illustrated atlas of the nerve tracts of the brain, cord, and peripheral nerves, including the autonomic nervous system. Each plate has an adequate descriptive legend.

\*University of California Hospital Formulary and Compendium of Useful Information. Cloth, 270 pages, \$2.00. University of California Press, Berkeley and Los Angeles, 1941.

†The American and His Food. A History of Food Habits in the United States. By Richard Osborn Cummings, Assistant Professor of History, Lawrence College. Cloth, 267 pages, \$2.50. University of Chicago Press, Chicago, Ill., 1940.

‡The March of Medicine. New York Academy of Medicine. Lectures to the Laity. Cloth, 154 pages, \$2.00. Columbia University Press, New York, N. Y., 1941.

§Essentials of Electrocardiography. By Richard Ashman, Ph.D., and Edgar Hull, M.D. Cloth, ed. 2, 373 pages, \$5.00. The Macmillan Company, New York, N. Y., 1941.

||Diagnostic Procedures and Reagents. Techniques for the Laboratory Diagnosis and Communicable Diseases. Cloth, 352 pages, \$2.75. American Public Health Association, 1700 Broadway, New York, N. Y., 1941.

¶The Principal Nervous Pathways—Neurological Charts and Schemas With Explanatory Notes. By Andrew Theodore Rasmussen, Ph.D., Professor of Neurology, Department of Anatomy, University of Minnesota Medical School, Minneapolis, Minn. Cloth, ed. 2, 73 pages. The Macmillan Company, New York, N. Y., 1941.

## Physical Medicine\*

THERE have been many volumes on physiotherapy. This is the first the reviewer has seen that appears to be fully authentic and free of overenthusiasm for one phase or another of physical therapy. It describes the methods of physical therapy as they are used at the Mayo Clinic. No section appears to receive undue emphasis. The author describes the application of heat and cold, light therapy, electrotherapy, diathermy, hydrotherapy, and mechanotherapy. A section deals with the application of the principles of physical therapy to various special diseases. Another section deals with the organization of physical therapy in hospitals. The introductory portion, which deals with the history of physical therapy, is especially interesting.

This volume will be useful not only to physiotherapists, but to others who wish to know the desirability of physical measures in special cases.

## Immunity Against Animal Parasites†

THE first volume dealing with this phase of medical study was Taliaferro's *Immunology of Parasitic Infections*, published in 1929. Culbertson's book takes up where Taliaferro left off, bringing the studies up to date. Part I deals with natural resistance and acquired immunity. Part II discusses immunity in specific diseases, such as amoebiasis, leishmaniasis, trypanosomiasis, malaria, coccidiosis, trematodiasis, cestodiasis, nematodiasis. The third section is on applied immunology; it classifies parasites and discusses preventive vaccination and diagnostic procedures.

Students of bacterial immunity will find much of interest in the volume. There is also much of practical service to clinicians.

## Pathology of the Oral Cavity‡

WRITTEN primarily for the dentist, this volume also contains sections on diseases of the jawbones and the oral mucosa. It is abundantly and well illustrated. All sections are short and concise. Emphasis is on diagnosis and significance of the lesions rather than upon therapeutic procedures.

## Practical Methods in Biochemistry§

A STUDENTS' laboratory manual used in the department of biochemistry at the University of Chicago. Although it will find its greatest use in student laboratory instruction, it will undoubtedly also serve well as a laboratory reference manual since it contains detailed instructions for the technique of the various clinical laboratory procedures. Especially valuable for reference purposes is the appendix in which fifty pages of small type are given to the preparation of standard reagents and dilutions.

\*Physical Medicine. By Frank H. Krusen, Foundation, University Clinic; Member of the C President of the American Physical Medicine. Cloth, 510 pages, with 100 illustrations. Philadelphia and London, 1941.

†Immunity Against Animal Parasites. By James T. Culbertson, Assistant Professor of Bacteriology, College of Physicians and Surgeons, Columbia University. Cloth, 274 pages, \$3.50. Columbia University Press, New York, N. Y., 1941.

‡Pathology of the Oral Cavity. By Lester Richard Cahn, D.D.S., Associate Professor of Dentistry (Oral Pathology), Columbia University, Fellow of the American Association for the Advancement of Science, Fellow of the New York Academy of Dentistry, Associate Fellow of the New York Academy of Medicine. The Williams & Wilkins Company, Baltimore, Md., 1941.

§Practical Methods in Biochemistry. By Frederick C. Koch, Frank P. Hixon Distinguished Service Professor of Biochemistry, University of Chicago. Cloth, ed. 3 (revised), 314 pages. A William Wood Book. The Williams & Wilkins Company, Baltimore, Md., 1941.

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## CLINICAL AND EXPERIMENTAL

### PULMONARY CEREBRAL EFFECT TIME AS DETERMINED BY AMYL NITRITE\*

#### I. ESTABLISHMENT OF A NORMAL RANGE

M. H. UHLEY, M.D., CHICAGO, ILL.

NUMEROUS methods have been devised for determining the rate of flow of blood from a given portion of the body to a distant designated locus. Since the work of Hering in 1827,<sup>1</sup> when he injected potassium ferrocyanide into one jugular vein of a horse, and tested samples of blood from the opposite jugular vein for Prussian blue, the greatest number of circulation time tests have involved the introduction of a chemical substance into a vein and the detection of that substance at some other point in the circulation by its objective or subjective manifestations.

Following in almost chronologic order is listed the evolution of circulation time studies: fluorescein<sup>2</sup>; calcium chloride<sup>3</sup>; concentrated salt solution producing distal electropotential changes<sup>4</sup>; radium C<sup>5-7</sup>; histamine flush reaction<sup>8, 17</sup>; phenoltetraiodophthalein sodium<sup>9</sup>; Congo red<sup>10</sup>; acetylcholine, determining volume changes in an extremity<sup>11</sup>; atropine and ephedrine, noting pupillary changes<sup>12</sup>; decholin<sup>13-15, 21</sup>; saccharine<sup>16</sup>; ether<sup>18</sup>; calcium gluconate<sup>19</sup>; magnesium sulfate, calcium gluconate, sodium chloride, copper sulfate mixture<sup>20</sup>; Kvale mixture,<sup>22</sup> and cyanide method.<sup>23</sup>

Other investigators have employed inhalation of various gases and have determined the rate of flow incidental to cardiac output studies, or the effect on the respiratory center; i.e., nitrous oxide method,<sup>24</sup> 5 to 7 per cent carbon dioxide inhalation,<sup>25</sup> rebreathing experiments with carbon dioxide,<sup>26</sup> ethyl iodide,<sup>27, 28</sup> and 50 per cent carbon dioxide inhalation.<sup>29</sup>

\*From the Cardiovascular Department, Michael Reese Hospital, Chicago.  
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All of the preceding, excepting the inhalation methods, necessitate single or double venipuncture. Those not relying upon subjective responses and their attendant variations, require in many instances complicated techniques or apparatus, making them almost prohibitive of widespread clinical application.

The method proposed in this report is readily controlled by a single operator, and obviates the use of venipuncture. It involves the inhalation of amyl nitrite, and the detection of drug action on the vessels of the head. The inhalation of amyl nitrite vapor is followed by its absorption into the pulmonary capillary bed, its transport to the left side of the heart, thence to the cerebral and peripheral vessels where objective and subjective phenomena may be noted. The arterial system of the head and neck is affected before the rest of the systemic circulation by virtue of the lesser distance to be traversed. Since the quantity of the drug is relatively small, and since the drug action is rapidly dissipated, peripheral systemic effects are seldom noted. A means is thus afforded for measuring the pulmonary cerebral effect time. Reasons for the use of this term will be made clear as the subject is developed in this report, which is limited to the establishment of a normal range of values for the method employed.

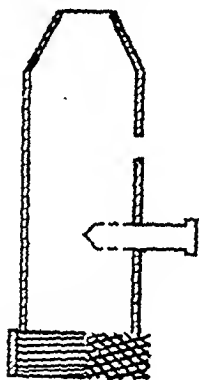


Fig. 1.—Inhaler.

#### APPARATUS AND TECHNIQUE

The amyl nitrite inhaler used for the test is a small cylindrical chamber (Fig. 1), 2 inches in length, with an inner diameter of  $\frac{1}{2}$  inch, and constructed of  $\frac{1}{16}$  inch aluminum tubing. It is fitted with a screw cap at one end, the opposite end tapering to an opening  $\frac{5}{16}$  inch in diameter. There are two  $\frac{1}{8}$  inch drill holes in the body. Into one of these is inserted a plunger with which to break the perles of amyl nitrite. A stop watch is the only other instrument necessary.

A 3 minim perle of amyl nitrite is placed in the chamber through the screw cap end, and the plunger is withdrawn. The operation is greatly facilitated by holding the chamber in the left hand, thumb at the screw cap end, middle finger over the opening at the tapered end, and index finger closing the first drill hole. The plunger is forced down with the right thumb, breaking the glass perle, its contents filling the chamber which is still sealed as described.

The patient is instructed that following a normal expiration, he is to inhale slowly and deeply from the inhaler (usually for 2 seconds) when it is brought to his nostril, and then to breathe normally again. The presence of abnormalities of the septum or turbinates is determined before this step, since an obstructed nasal passage would not permit inhalation of the drug. He is told that the medication inhaled will produce a "new feeling" or "new sensation" in his head, which will appear as a slight dizziness or fullness, followed by a feeling of warmth in the face and neck. The entire reaction will disappear within one to three minutes. The patient is asked to signal the observer immediately upon perception of any sensory change. The signal, previously agreed upon, consists either in raising the hand or saying "now." The starting time for the test is the time at which inhalation is completed, and the end point is the time indicated by the signal.

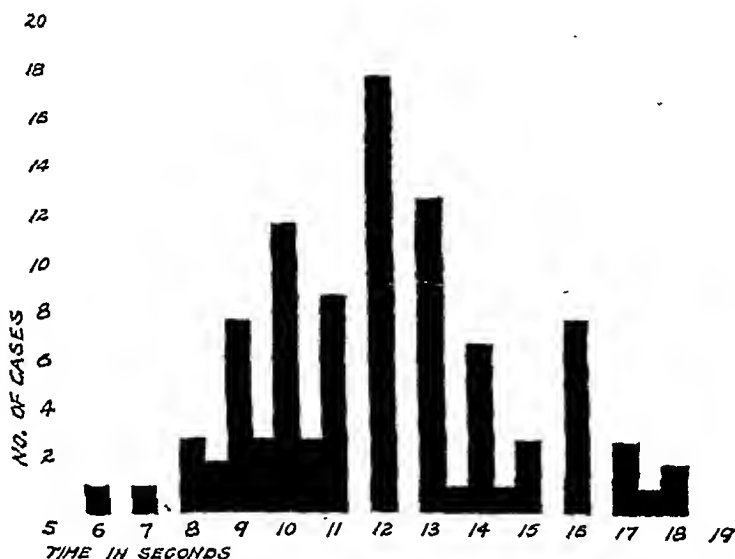


Fig. 2.—Pulmonary cerebral effect time in 100 cases.

## RESULTS

One hundred persons selected at random from the interne, nursing, and house staff of Michael Reese Hospital were tested by the advocated method, and the results were noted. They are assembled in a graph form, shown in Fig. 2.

The greater majority of measurements lie between 10 and 16 seconds. The average time for the 100 cases is twelve seconds. This spread is no greater than that obtained by other methods currently in use.

In the proposed method for determining pulmonary cerebral effect time, the first manifestations are noted in the distribution of the external and internal carotid arteries. Occasionally, throbbing in the arms, and perspiration and warmth of the hands are later noted. A few persons have coughed after the full effect has been reached.

The only untoward reaction found in the series, from the use of amyl nitrite in the dose administered, was the presence of headache, persisting from ten minutes to three hours in three persons.

## DISCUSSION

A number of factors must be given consideration in evaluating any circulation study. As a matter of fact, no result may be properly called "circulation time," unless objective and mechanically recorded intervals along the course of a given segment of the vascular tree being investigated are noted. Any instance in which a sensory response is taken as an end point lends itself to errors of latency in perception and expression. There is another phase of rate of flow studies that is not sufficiently emphasized. In those techniques involving passage of a test medium through the lung (e.g., inhalation and ether), there is an inherent and quite variable "across membrane" delay in that structure. Thus it is obvious that a more accurate term encompassing flow rate and end point manifestation is desirable. The term "effect time" is suggested for this purpose.

The possibility exists that amyl nitrite changes the circulatory tree through which it passes. The reaction of amyl nitrite has been investigated by Lander Brunton in 1871, who noted its pronounced but transient arterial dilatation, attributing the phenomenon to a direct effect upon the smooth muscle of the blood vessels, independent of any nervous mechanism.<sup>30</sup> His investigations have been verified by innumerable subsequent investigators, who found that cerebral vessels,<sup>31</sup> pulmonary vessels,<sup>32</sup> coronary vessels,<sup>33</sup> splanchnics,<sup>34</sup> and peripheral vessels,<sup>35</sup> respond by dilatation. Pilcher and Sollman in 1915<sup>36</sup> showed that the vasomotor center is not influenced directly by the drug when they observed that there was no blood pressure fall or rise following introduction of amyl nitrite only into the cerebral circulation. Vagal depression occurs only as a compensatory response to the systemic fall in pressure, and is, therefore, not due to an isolated action of the drug. This was adequately demonstrated by Filehne,<sup>37</sup> who showed that when the pressure was kept level by partial clamping of the aorta, no increase in rate occurred.

The time interval between the inhalation and manifestation of drug action is dependent upon two main factors, aside from the reaction time, assuming that the dose is fairly uniform and the technique is constant: (1) circulatory dynamic factor; (2) respiratory dynamic factor.

The first of these factors, i.e., the circulatory dynamic factor, refers more specifically to those changes associated with the occurrence of cardiac failure and concomitant slowing of the circulation. The test method can be interpreted as an index of the circulation of the left side of the heart, since the drug enters the circulation at the lung capillaries and has its effect after passing through the blood vessels of the head. It does not measure rate of circulation through the right side of the heart.

However, the second factor, the respiratory dynamic factor, comes into play. Alterations in the pulmonary ventilation, total capacity, or alveolar transport, will affect the measurement. Thus chronic pulmonary diseases, such as emphysema, bronchitis, bronchiectasis, and bronchial asthma, can affect the results noted.

The pulmonary cerebral effect time thus may be viewed as an additive phenomenon. Consequently, before a result is ascribed to an altered state of circula-



tion of the left side of the heart, the effects of pulmonary disease and abnormal reaction times must be considered in each case individually.

Furthermore, the circulation time itself is determined not only by the minute volume output of the heart, but also by the quantity of blood contained in the path from the lung capillaries to the head circuit. This is usually most variable in the pulmonary capillaries and veins, and next in the left auricle.

In short, amyl nitrite is a drug whose prime action is limited to a direct effect upon the vascular system, probably not associated with reflex dilatation or constriction. It may be used as a clinical test as suggested, even though the end point is a subjective one, necessitating recognition of the variations in individual reaction time. The discomfort associated with its use is relatively mild and disappears rapidly.

There is perhaps a single contraindication to the use of amyl nitrite, i.e., the presence of glaucoma, for as shown by Wessely,<sup>38</sup> it causes an increase in intra-ocular tension in spite of the lowering of systemic pressure.

#### CONCLUSIONS

A simple method for quantitating pulmonary cerebral effect time is described, which measures the effective pulmonary ventilation, the circulation time of the left side of the heart, and is affected by variations in the reaction time of the subject.

The normal values in 100 cases are presented.

Its use in determining the degree of failure of circulation of the left side of the heart circuit is thus limited to instances in which no disease of the pulmonary apparatus exists, and in which the reaction time of the subject is not abnormal. Conversely, it may offer an index of the degree of diffuse chronic pulmonary disease in the absence of cardiac disease. When abnormalities are present in both pulmonary and vascular systems, the combined effects may be measured, but without evaluation of the component parts.

I wish to thank Dr. L. N. Katz, under whose supervision this work was done, for suggestions made during the study and preparation of this report.

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## REGULATION OF THE RECIPROCAL ACTIVITY OF DESOXYCORTICOSTERONE ACETATE AND SODIUM IN ADDISON'S DISEASE\*

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THE early failure to understand the extent to which the physiologic activity of desoxycorticosterone acetate is dependent upon the sodium available in the organism has accounted for most of the complications following its administration to patients with Addison's disease. Six patients with this malady have been treated with the drug† over periods varying from three months to two and one-half years, during which time in each instance there have been wide variations in the amounts of sodium and desoxycorticosterone acetate employed. Patients were judged to be in a state of "adrenal sufficiency," that is, in good clinical condition, when a sense of well-being was subjectively experienced; a gradual gain in weight to normal and its maintenance achieved; a return of blood pressure to satisfactory levels attained; a cardiothoracic ratio between 0.45 and 0.50 observed; and normal values for sodium and potassium noted in both blood serum and urine.

By "trial and error" sodium and desoxycorticosterone acetate have been utilized in 47 different ratios in the above-mentioned six patients for not less than six weeks in any instance, unless severe insufficiency or a complication arose before such a period was completed. The longest time at any single dose level in any one individual has been nine months. When these average daily values for sodium ingested and desoxycorticosterone acetate administered were plotted against each other, it was found that patients in good clinical condition showed a definite reciprocal relationship between sodium intake and desoxycorticosterone acetate requirement which was capable of mathematical expression in terms of hyperbolic curves. Seventy-one per cent of these satisfactory observations lay on or between curves, the constants of which were 37.5 and 45.0, respectively (Fig. 1), while all other observations (29 per cent) in which the clinical condition of the patient was satisfactory were represented by similar parabolic curves, the constants of which ranged from 30 to 37.5, and from 45 to 55, respectively (Fig. 1.)

It is obvious that only portions of the hyperbolas are of practical value. If one uses large quantities of sodium, that is, more than 9 Gm. daily, and correspondingly small doses of desoxycorticosterone acetate, the clinical condition is not as good as though more desoxycorticosterone acetate were employed. If, on

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the other hand, sodium is severely restricted, that is, to 1.5 Gm., and desoxycorticosterone acetate dosage is concomitantly increased, that is, to 30 mg. daily, some "packing" of sodium into the cells probably occurs.<sup>1, 2</sup>

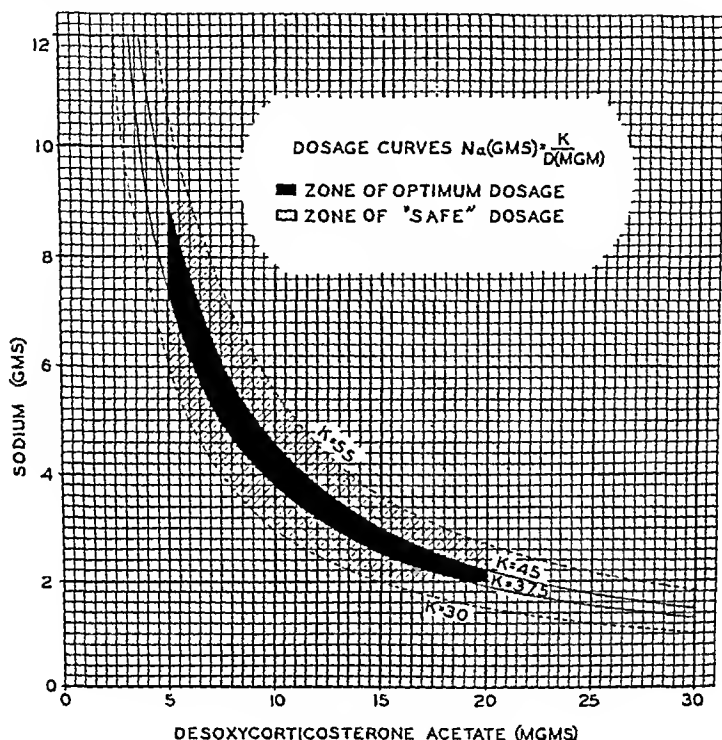


Fig. 1.

The formula  $Na \text{ (Gm.)} = \frac{k}{D \text{ (mg.)}}$ , where Na equals daily intake of sodium in grams, k is a constant, and D the desoxycorticosterone acetate in milligrams, is applicable to the management of patients with Addison's disease over a definite range of values, suggested by the shaded area in Fig. 1. In our experience, values for the daily intake of sodium should not exceed 9 Gm. or be less than 2 Gm., while less than 5 mg. or more than 20 mg. of desoxycorticosterone acetate daily do not afford as satisfactory relief as those attendant upon amounts between these two figures. Values for k should lie on or between 37.5 and 45. The area delimited by adherence to these figures (black in Fig. 1) may be spoken of as the "area of optimum dosage" in the use of desoxycorticosterone acetate for the management of Addison's disease. Values lying within this space can be apparently applied without danger of disaster from cortical insufficiency or fear of complications directly related to the therapy.

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# THE SIGNIFICANCE OF FIBRINOLYSIS IN MECHANISM OF COAGULATION OF BLOOD\*

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BY FIBRINOLYSIS is meant an occasionally observed phenomenon which consists in the dissolution of the clot of fibrin formed by the coagulation of whole blood or plasma. This phenomenon has been observed and described by several authors, principally Denis (quoted by Nolf<sup>11</sup>), Dastre,<sup>2</sup> Rulot,<sup>19, 20</sup> Nolf,<sup>11</sup> Hougardy,<sup>4, 5</sup> and Hirose.<sup>18</sup> Since fibrinolysis appears only occasionally in the process of blood coagulation, it is necessary to devise some procedure for obtaining it consistently, in order to be able to investigate it on an experimental basis. The use of chloroform provides such a procedure, as will be shown by the subsequent considerations.

The effect of chloroform on blood coagulation has been investigated principally by Howell,<sup>6</sup> Nolf,<sup>9, 14</sup> Minot,<sup>7</sup> and Bordet.<sup>1</sup> All these authors agree that this substance has a marked effect on the mechanism of blood coagulation; they have observed that chloroform was able to clot oxalated plasma. Nolf emphasized the fibrinolytic power of the serum obtained from oxalated recalcified plasma clotted by chloroform.

As far as fibrinolytic action is concerned, the best procedure to study the effect of chloroform is to observe the serum resulting from oxalated, recalcified plasma clotted by chloroform. Blood is taken by venipuncture from the jugular vein of an unanesthetized, fasting dog. Twenty cubic centimeters of blood are withdrawn in a syringe containing 1 c.c. of 3 per cent sodium oxalate solution. It is then poured into a paraffined centrifuge tube and is centrifuged twice for fifteen minutes at a speed of 3,000 r.p.m. The clear plasma is used to prepare:

1. Normal serum, by simply adding calcium chloride. After coagulation occurs, serum is obtained by centrifugalization.

2. So-called chloroform serum. Recalcified plasma is mixed with  $\frac{1}{10}$  its volume of chloroform and is shaken for one minute in a carefully stoppered tube. The tube is maintained at a temperature of 37° C. for one hour. Then chloroform is removed from the mixture, first by centrifugalization and then by evaporation of its supernatant layer in the icebox for sixteen to twenty-four hours. After that time the mixture does not smell of chloroform. Distilled water is added to correct for the loss of water caused by evaporation. The serum is filtered before using.

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In the course of this study, other reagents were used:

*Oxalated plasma*: as such, or deprived of fibrinogen by heating at 56° C. for two minutes.

*Prothrombin*: globulin solution prepared from diluted oxalated plasma, precipitated at pH 5.3, following the method of Mellanby.<sup>8</sup> It can be deprived of fibrinogen by heating to 56° C.

*Fibrinogen*: prepared according to Warner, Brinkhous, and Smith.<sup>23</sup> It was diluted with physiologic saline before being used, until the greatest dilution was obtained which gave, with the addition of thrombin, a coherent clot. This dilution is desirable because it decreases the amount of impurities remaining in the solution.

*Thromboplastin*: prepared according to Warner, Brinkhous, and Smith.<sup>23</sup>

*Oxalated plasma deprived of prothrombin*: To remove prothrombin, tricalcium phosphate was used in a thick creamy suspension in saline. Ten parts of oxalated plasma are added with one part of the suspension. After careful stirring, the mixture is warmed to 37° C. for fifteen minutes, and then the phosphate, together with the prothrombin, is removed by centrifugalization. Phosphated plasma does not clot on addition of calcium and thromboplastin. It clots readily on addition of thrombin. It can be deprived of fibrinogen by heating to 56° C.

*Thrombin*: prepared just before its use by adding calcium and thromboplastin to oxalated plasma or to prothrombin and removing the serum after coagulation.

All the foregoing blood preparations were made from dog blood.

To compare the thrombin content of different sera, successive dilutions of the sera were added to given quantities of fibrinogen. The most dilute amount which produces a clot and thereby contains an appreciable, effective quantity of thrombin is taken as the end point. Sera prepared by various methods—chloroform serum, normal serum—may thus be compared for thrombin activity.

In all the experiments a total volume of 1 c.c. was used. Physiologic saline was used as a diluent. In order to keep the quantity of thrombin tested at a constant known level, an excess of sodium oxalate was added to prevent further increment of thrombin by conversion of any prothrombin which might possibly be present. The experiments were performed at room temperature. Under these conditions the amount of thrombin in chloroform serum and normal serum was compared. Such a comparison is given in Table I.

It appears that chloroform serum contains 300 times as much thrombin as normal serum. Table I shows also that chloroform serum, after having clotted fibrinogen, dissolves it. In the first tube (Table I), the fibrinogen clots in four minutes. The following day the clot had disappeared. The solution was again perfectly clear, and its appearance was absolutely the same as before the occurrence of the clot. An important change, however, had occurred. The solution contained no more fibrinogen. It was impossible to obtain a clot in this solution by adding thrombin. Also, if the content of the first tube is heated to 56° C., fibrinogen precipitate does not appear. The same phenomenon oc-

curred in the two following tubes (Nos. 2 and 3). It occurred in the fourth tube (No. 4), but less intensively. This did not occur with normal serum. The disappearance of the clot is probably due to proteolysis.

TABLE I

COMPARISON OF THE THROMBIN CONTENT OF CHLOROFORM SERUM AND NORMAL SERUM

| TUBES | REAGENTS IN CUBIC CENTIMETERS |               |                 |                          |                 | RESULTS  |
|-------|-------------------------------|---------------|-----------------|--------------------------|-----------------|--|
|       | SODIUM<br>OXALATE<br>1%       | NaCl<br>0.90% | FIBRINO-<br>GEN | CHLORO-<br>FORM<br>SERUM | NORMAL<br>SERUM |  |
| 1     | 0.1                           | 0.7           | 0.1             | 0.1                      |                 | Clot in 4 min. Redissolved after 16 hr.            |
| 2     | 0.1                           | 0.7           | 0.1             | 0.01                     |                 | Clot in 10 min. Redissolved after 8 min.           |
| 3     | 0.1                           | 0.7           | 0.1             | 0.003                    |                 | Clot in 10 min. Redissolved after 20 min.          |
| 4     | 0.1                           | 0.7           | 0.1             | 0.001                    |                 | Clot in 30 min. Half dissolved after 2 hr. 20 min. |
| 5     | 0.1                           | 0.7           | 0.1             | 0.0003                   |                 | Clot in 1 hr. 10 min. Not redissolved              |
| 6     | 0.1                           | 0.7           | 0.1             | 0.0001                   |                 | Clot in 2 hr. 7 min. Not redissolved               |
| 7     | 0.1                           | 0.7           | 0.1             | 0.00003                  |                 | Clot in 3 hr. 49 min. Not redissolved              |
| 8     | 0.1                           | 0.7           | 0.1             | 0.00001                  |                 | Clot in 10 hr. 57 min. Not redissolved             |
| 9     | 0.1                           | 0.7           | 0.1             |                          | 0.1             | Clot in 1 hr. 10 min. Not redissolved              |
| 10    | 0.1                           | 0.7           | 0.1             |                          | 0.01            | Clot in 1 hr. 10 min. Not redissolved              |
| 11    | 0.1                           | 0.7           | 0.1             |                          | 0.003           | Clot in 2 hr. 20 min. Not redissolved              |
| 12    | 0.1                           | 0.7           | 0.1             |                          | 0.001           | Clot in 12 hr. Not redissolved                     |
| 13    | 0.1                           | 0.7           | 0.1             |                          | 0.0003          | Clot in 12 hr. Not redissolved                     |
| 14    | 0.1                           | 0.7           | 0.1             |                          | 0.0001          | No clot  |

If the action of chloroform serum on diluted oxalated plasma is studied, it appears that a solution of the clot does not occur in plasma as markedly as in the fibrinogen solution. Table II shows that dilutions of chloroform serum, which are able to produce fibrinolysis in the fibrinogen solution, do not produce this when acting on diluted plasma. It can be seen in Table III that the quantity of plasma needed to prevent fibrinolysis caused by 0.1 c.c. of chloroform serum is between 0.04 c.c. and 0.01 c.c. (tube 6). The preventive action of plasma on proteolysis is not due to fibrinogen, since this inhibiting effect remains unchanged when plasma is deprived of fibrinogen by heating to 56°. This is apparent in Table IV. It will be shown later that the preventive action is due to the prothrombin of the plasma (Tables IX and X).

In the experiment described above, 0.01 c.c. of chloroform serum causes lysis of the fibrinogen clot after four minutes. Fibrinolysis may occur at an even more rapid rate, and in such cases a well-formed clot would have no time to appear. Table V illustrates such an experiment (tube 2). It can be seen that 0.1 c.c. of chloroform serum causes the production of flakes in the fibrinogen solution. These flakes disappear after two minutes, and thereafter there is no more fibrinogen in the solution. This shows that fibrinolysis may be completed

TABLE II

COMPARATIVE ACTION OF CHLOROFORM SERUM ON FIBRINOGEN AND OXALATED PLASMA

| TUBES | REAGENTS IN CUBIC CENTIMETERS |              |                 |                    |                          | RESULTS                                |
|-------|-------------------------------|--------------|-----------------|--------------------|--------------------------|--|
|       | SODIUM<br>OXALATE<br>1%       | NaCl<br>0.9% | FIBRINO-<br>GEN | OXALATED<br>PLASMA | CHLORO-<br>FORM<br>SERUM |  |
| 1     | 0.1                           | 0.7          | 0.1             |                    | 0.1                      | Clot in 2 min. Redissolved after 3 hr. |
| 2     | 0.1                           | 0.7          | 0.1             |                    | 0.01                     | Clot in 2 min. Redissolved after 3 hr. |
| 3     | 0.1                           | 0.7          | 0.1             |                    | 0.003                    | Clot in 2 min. Not redissolved         |
| 4     | 0.1                           | 0.7          |                 | 0.1                | 0.1                      | Clot in 2 min. Not redissolved         |
| 5     | 0.1                           | 0.7          |                 | 0.1                | 0.01                     | Clot in 2 min. Not redissolved         |
| 6     | 0.1                           | 0.7          |                 | 0.1                | 0.003                    | Clot in 2 min. Not redissolved         |

TABLE III

PREVENTIVE ACTION OF PLASMA ON FIBRINOLYSIS CAUSED BY CHLOROFORM SERUM

| TUBES | REAGENTS IN CUBIC CENTIMETERS |              |                 |                         |                          | RESULTS                                |
|-------|-------------------------------|--------------|-----------------|-------------------------|--------------------------|--|
|       | SODIUM<br>OXALATE<br>1%       | NaCl<br>0.9% | FIBRINO-<br>GEN | OX-<br>ALATED<br>PLASMA | CHLORO-<br>FORM<br>SERUM |  |
| 1     | 0.1                           | 0.5          | 0.1             | 0.2                     | 0.1                      | Clot in 5 min. Not redissolved         |
| 2     | 0.1                           | 0.6          | 0.1             | 0.1                     | 0.1                      | Clot in 5 min. Not redissolved         |
| 3     | 0.1                           | 0.3          | 0.1             | 0.08                    | 0.1                      | Clot in 5 min. Not redissolved         |
| 4     | 0.1                           | 0.4          | 0.1             | 0.06                    | 0.1                      | Clot in 5 min. Not redissolved         |
| 5     | 0.1                           | 0.5          | 0.1             | 0.04                    | 0.1                      | Clot in 5 min. Not redissolved         |
| 6     | 0.1                           | 0.6          | 0.1             | 0.01                    | 0.1                      | Clot in 5 min. Redissolved after 3 hr. |

TABLE IV

ACTION OF PLASMA, DEPRIVED OF FIBRINOGEN, ON FIBRINOLYSIS

| TUBES | REAGENTS IN CUBIC CENTIMETERS |              |                 |  |                          | RESULTS                                |
|-------|-------------------------------|--------------|-----------------|--|--------------------------|--|
|       | SODIUM<br>OXALATE<br>1%       | NaCl<br>0.9% | FIBRINO-<br>GEN | OXALATED<br>PLASMA<br>DEPRIVED<br>OF FI-<br>BRINOGEN | CHLORO-<br>FORM<br>SERUM |  |
| 1     | 0.1                           | 0.5          | 0.1             | 0.2  | 0.1                      | Clot in 5 min. Not redissolved         |
| 2     | 0.1                           | 0.6          | 0.1             | 0.1  | 0.1                      | Clot in 5 min. Not redissolved         |
| 3     | 0.1                           | 0.3          | 0.1             | 0.08   | 0.1                      | Clot in 5 min. Not redissolved         |
| 4     | 0.1                           | 0.4          | 0.1             | 0.06   | 0.1                      | Clot in 5 min. Not redissolved         |
| 5     | 0.1                           | 0.5          | 0.1             | 0.04   | 0.1                      | Clot in 5 min. Not redissolved         |
| 6     | 0.1                           | 0.6          | 0.1             | 0.01   | 0.1                      | Clot in 5 min. Redissolved after 3 hr. |

TABLE V

FIBRINOLYSIS OCCURRING IMMEDIATELY AFTER COAGULATION

| TUBES | REAGENTS IN CUBIC CENTIMETERS |              |                 |                          | RESULTS                                      |
|-------|-------------------------------|--------------|-----------------|--------------------------|--|
|       | SODIUM<br>OXALATE<br>1%       | NaCl<br>0.9% | FIBRINO-<br>GEN | CHLORO-<br>FORM<br>SERUM |  |
| 1     | 0.1                           | 0.6          | 0.1             | 0.2                      | No change                                    |
| 2     | 0.1                           | 0.7          | 0.1             | 0.1                      | Flakes after 3 min. Redissolved after 2 min. |
| 3     | 0.1                           | 0.7          | 0.1             | 0.01                     | Clot in 5 min. Redissolved after 2 hr.       |



after two minutes. It may even occur so soon that the coagulation could not be observed. The solution of fibrinogen to which chloroform serum is added becomes opalescent and then clear again. No compact clot is formed. The solution, however, contains no more fibrinogen. This phenomenon is illustrated in Table V (tube 1). Nothing happens in the tube to which 0.2 c.c. of chloroform serum is added, but in the tubes to which is added 0.01 c.c. of the serum, coagulation occurs, soon followed by proteolysis. In the first tube, fibrinogen disappears, fibrinolysis having set in so fast that the fibrin flakes, if formed at all, are dissolved as soon as they are formed. At this dilution chloroform serum seems to have no clotting power. Moreover, a superficial observation would lead one to believe that it has an ant clotting power. However, this is not so, since fibrinogen originally present has disappeared. It is, therefore, impossible to obtain coagulation in the first tube. The disappearance of fibrinogen is the real cause for the incoagulability. Moreover, this interpretation is confirmed by the fact that the same quantity of serum, which produces no apparent coagulation in the fibrinogen, does clot oxalated diluted plasma. Fibrinolysis does not occur in plasma as it does in the fibrinogen mixture, probably because of some anti-lytic agents in the former.

Fibrinolysis usually occurs without apparent coagulation if serum kept in contact with chloroform for twenty-four hours is used. This is not usually the case with serum kept in contact with chloroform for only one hour. The fibrinolytic property is increased when the contact time with chloroform is prolonged, provided that this time does not exceed forty-eight hours (Table VI).

TABLE VI

COMPARISON OF THE FIBRINOLYTIC POWER OF CHLOROFORM SERUM AFTER ONE HOUR OF CONTACT WITH CHLOROFORM AND AFTER TWENTY-FOUR HOURS

| TUBES | SODIUM<br>OXALATE<br>1% | NaCl<br>0.9% | FIBRINO-<br>GEN | CHLOROFORM<br>SERUM<br>AFTER ONE<br>HOUR OF<br>CONTACT | CHLOROFORM<br>SERUM<br>AFTER 24<br>HOURS OF<br>CONTACT | RESULTS  |
|-------|-------------------------|--------------|-----------------|--|--|--|
| 1     | 0.1                     | 0.7          | 0.1             | 0.1  |  | Clot in 5 min. Redissolved<br>after 10 min.      |
| 2     | 0.1                     | 0.7          | 0.1             | 0.01   |  | Clot in 10 min. Redissolved<br>after 30 min.     |
| 3     | 0.1                     | 0.7          | 0.1             | 0.001  |  | Clot in 30 min. Not redis-<br>solved             |
| 4     | 0.1                     | 0.7          | 0.1             |  | 0.1  | No change (proteolysis with-<br>out coagulation) |
| 5     | 0.1                     | 0.7          | 0.1             |  | 0.01   | No change (proteolysis with-<br>out coagulation) |
| 6     | 0.1                     | 0.7          | 0.1             |  | 0.001  | Clot in 45 min. Not redis-<br>solved             |

In order to investigate further the relationship of fibrinolysis to the mechanism of blood coagulation, chloroform serum produced by allowing serum to remain in contact with chloroform for one hour at 37° C. and for twenty-four hours at room temperature, was used in the following experiments. Hereafter the expression "chloroform serum" will always refer to this particular twenty-four-hour-old serum.

It has been shown (Table V) that oxalated plasma contains antilytic substances. The substances contained in plasma, however, are not the only ones showing an antilytic effect. Thromboplastin exhibits it also. It can be seen in Table VII (tube 3) that 0.1 e.c. of thromboplastin, diluted ten times, counteracts the fibrinolytic effect of a quantity of chloroform serum able to fibrinolyze the fibrinogen without producing clot formation (tube 5). Consequently, if two identical mixtures of oxalated fibrinogen and oxalated chloroform serum are used, thromboplastin added to one of them will cause a clot to appear while the other one remains fluid and undergoes fibrinolysis without coagulation. This antilytic effect of thromboplastin is very powerful.

TABLE VII  
ANTILYTIC EFFECT OF THROMBOPLASTIN

| TUBES | OXALATED<br>CHLORO-<br>FORM<br>SERUM | NaCl<br>0.9% | OX-<br>ALATED<br>FIBRINO-<br>GEN | THROMBO-<br>PLASTIN<br>(DILUTED 10<br>TIMES) | RESULTS                                 |
|-------|--------------------------------------|--------------|----------------------------------|--|---|
| 1     | 0.1                                  | 0.5          | 0.1                              | 0.3  | Clot in 5 min. Not redissolved.         |
| 2     | 0.1                                  | 0.6          | 0.1                              | 0.2  | Clot in 5 min. Not redissolved.         |
| 3     | 0.1                                  | 0.7          | 0.1                              | 0.1  | Clot in 5 min. Redissolved after 10 hr. |
| 4     | 0.1                                  | 0.7          | 0.1                              | 0.03   | Proteolysis without coagulation         |
| 5     | 0.1                                  | 0.8          | 0.1                              | 0  | Proteolysis without coagulation         |

In order to ascertain more accurately the fibrinolytic action of chloroform serum, the following procedure was undertaken: Chloroform serum was diluted with 15 volumes of distilled water and, with the use of N/10 acetic acid, mixtures of varying pH were obtained. The globulin precipitate obtained in every case was dissolved in saline, and its action was tested on fibrinogen and oxalated plasma. Only the product obtained at pH 6 showed any activity comparable with that of the chloroform serum which produced it. In addition, it revealed some interesting properties which explain the action of chloroform serum.

The detailed method of preparation was the following: Chloroform serum is diluted with 15 volumes of cold, distilled water in order to lower the salt concentration below 1 per thousand. Decinormal acetic acid is added until pH 6 is reached. The slightly turbid solution is centrifuged, and the precipitate is dissolved in a volume of saline equal to one-third that of the chloroform serum from which it was obtained. Sodium oxalate is added in order to obtain a concentration of soluble oxalate of 0.15 per cent. The solution is centrifuged, and its pH is adjusted to neutrality. This solution is called *solution A* in the experiments to be detailed.

The properties of solution A are:

1. It is not a solution of thrombin, since at no concentration does it clot fibrinogen. Unlike chloroform serum, of which a concentration can always be found which clots fibrinogen and dissolves it afterwards, every active concentration of solution A fibrinolyses fibrinogen without clotting it. Dilute quantities of solution A leave it unchanged. Table VIII shows the difference in the effects of chloroform serum and of solution A (prepared from the chloroform serum) on fibrinogen.

TABLE VIII

COMPARISON OF THE EFFECT OF CHLOROFORM SERUM AND OF SOLUTION A ON FIBRINOGEN

| TUBES | OXALATED<br>CHLORO-<br>FORM<br>SERUM | OXALATED<br>SOLUTION<br>A | OXALATED<br>FIBRINO-<br>GEN | NaCl<br>0.9% | RESULTS                                   |
|-------|--------------------------------------|---------------------------|-----------------------------|--------------|---|
| 1     | 0.1                                  |                           | 0.1                         | 0.7          | Proteolysis without coagulation           |
| 2     | 0.01                                 |                           | 0.1                         | 0.7          | Proteolysis without coagulation           |
| 3     | 0.001                                |                           | 0.1                         | 0.7          | Clot in 20 min. Redissolved after 10 min. |
| 4     | 0.0001                               |                           | 0.1                         | 0.7          | No clot. No proteolysis                   |
| 5     | 0.00001                              |                           | 0.1                         | 0.7          | No clot. No proteolysis                   |
| 6     |                                      | 0.4                       | 0.1                         | 0.5          | Proteolysis without coagulation           |
| 7     |                                      | 0.2                       | 0.1                         | 0.7          | Proteolysis without coagulation           |
| 8     |                                      | 0.1                       | 0.1                         | 0.8          | Proteolysis without coagulation           |
| 9     |                                      | 0.06                      | 0.1                         | 0.7          | No clot. No proteolysis                   |
| 10    |                                      | 0.03                      | 0.1                         | 0.7          | No clot. No proteolysis                   |

TABLE IX

ACTION OF SOLUTION A ON OXALATED PLASMA

| TUBES | OXALATED<br>PLASMA<br>DEPRIVED<br>OF FI-<br>BRINOGEN | OXALATED<br>FIBRINO-<br>GEN | OXALATED<br>SOLUTION<br>A | NaCl<br>0.9% | RESULTS                                 |
|-------|--|-----------------------------|---------------------------|--------------|---|
| 1     | 0.1  | 0.1                         | 0.5                       | 0.3          | Clot in 15 min. Redissolved after 2 hr. |
| 2     | 0.08   | 0.1                         | 0.5                       | 0.1          | Clot in 15 min. Redissolved after 1 hr. |
| 3     | 0.06   | 0.1                         | 0.5                       | 0.2          | Proteolysis without coagulation         |
| 4     | 0.04   | 0.1                         | 0.5                       | 0.3          | Proteolysis without coagulation         |
| 5     | 0.02   | 0.1                         | 0.5                       | 0.3          | Proteolysis without coagulation         |

TABLE X

ACTION OF SOLUTION A ON PHOSPHATED PLASMA

| TUBES | REAGENTS IN CUBIC CENTIMETERS |              |                        | RESULTS                         |
|-------|-------------------------------|--------------|------------------------|---------------------------------|
|       | PHOSPHATED<br>PLASMA          | NaCl<br>0.9% | OXALATED<br>SOLUTION A |                                 |
| 1     | 0.1                           | 0.1          | 0.4                    | Proteolysis without coagulation |
| 2     | 0.1                           | 0.3          | 0.2                    | Proteolysis without coagulation |
| 3     | 0.1                           | 0.5          | 0.05                   | No proteolysis. No coagulation  |
| 4     | 0.1                           | 0.7          | 0.01                   | No proteolysis. No coagulation  |

TABLE XI

ACTION OF SOLUTION A ON FIBRINOGEN TO WHICH THROMBOPLASTIN IS ADDED

| TUBES | OXALATED<br>FIBRINO-<br>GEN | THROMBO-<br>PLASTIN | OXALATED<br>SOLUTION<br>A | NaCl<br>0.9% | RESULTS                         |
|-------|-----------------------------|---------------------|---------------------------|--------------|---------------------------------|
| 1     | 0.1                         | 0.02                | 0.4                       | 0.3          | Proteolysis without coagulation |
| 2     | 0.1                         | 0.01                | 0.4                       | 0.4          | Proteolysis without coagulation |
| 3     | 0.1                         | 0.003               | 0.4                       | 0.4          | Proteolysis without coagulation |
| 4     | 0.1                         | 0.001               | 0.4                       | 0.4          | Proteolysis without coagulation |

2. Added to oxalated plasma, solution A has the same action as chloroform serum. It clots oxalated plasma, provided there is a sufficient amount of the latter (Table IX, tubes 1 and 2). If the quantity of plasma is too small, solution A fibrinolyses fibrinogen without clotting it, as shown in Table IX, tube 3.

3. Added to oxalated plasma deprived of prothrombin (phosphated plasma), solution A never clots it, but added in sufficient quantity solution A fibrinolyses the fibrinogen of phosphated plasma (Table X, tubes 1 and 2). Added in smaller quantities, it leaves it intact, as shown in Table X, tubes 3 and 4.

4. If thromboplastin is added to fibrinogen, solution A produces no clot, in contrast to chloroform serum the fibrinolytic power of which, as stated previously, can be transformed into clotting power by means of thromboplastin (Table XI).

5. From the foregoing experiments it may be concluded that solution A is not prothrombin. Prothrombin has no clotting effect in the absence of calcium.

6. Solution A is not thromboplastin because thromboplastin never clots oxalated plasma.

In summary, it may be said that solution A has an enzymelike, and probably a proteolytic effect on fibrinogen. Its activity, with respect to the phenomenon of blood coagulation, presents the following features: it clots oxalated plasma. Unlike chloroform serum, its mother substance, solution A never clots plasma deprived of prothrombin, nor does it coagulate fibrinogen to which thromboplastin is added. The clotting activity of solution A is probably due to its action on prothrombin, which is converted into thrombin even in the presence of an excess of sodium oxalate without participation of calcium platelets or thromboplastin.

There is a striking similarity of action between the enzymelike substance contained in solution A and trypsin. The effect of trypsin on blood coagulation was studied principally by Eagle and Harris.<sup>3</sup> According to these authors, trypsin, like solution A, lyzes fibrinogen without clotting it, but it has a strong clotting action on oxalated plasma without the participation of calcium, platelets, or thromboplastin. Added to prothrombin, optimum quantities of trypsin change it into thrombin. Larger quantities fail to do so since they bring about the destruction of prothrombin. The same thing happens when trypsin is allowed to remain in contact with prothrombin for a long time. Both prothrombin and the thrombin which is formed are destroyed. Eagle and Harris conclude that trypsin acts in the mechanism of blood coagulation like the system calcium platelets or calcium thromboplastin does. The theory of blood coagulation, adopted by Eagle and Harris, according to which thrombin results from the interaction between prothrombin and thromboplastin plus calcium, permits no other conclusion.

In order to investigate further the identity of the action of trypsin and the enzyme contained in solution A, the effect of solution A on the prothrombin solution, prepared as described above, was studied. Varying quantities of solution A were added to 0.5 c.c. of oxalated prothrombin. They were allowed to

interact at 37° C. during different periods of time. The thrombin produced in every case was tested on fibrinogen, and the clotting time was recorded. To rule out an eventual spontaneous formation of thrombin from prothrombin, a control was made by allowing pure prothrombin to remain at 37° C. for the time of the experiment, and testing it on fibrinogen. The experiment was carried out at pH 6.7, 0.5 e.e. of phosphate buffer being added to each sample. The results (Chart 1) show that the activation of prothrombin into thrombin by solution A is soon followed by inactivation of both the prothrombin and the thrombin formed. This is consistent with the results of Eagle and Harris with trypsin.

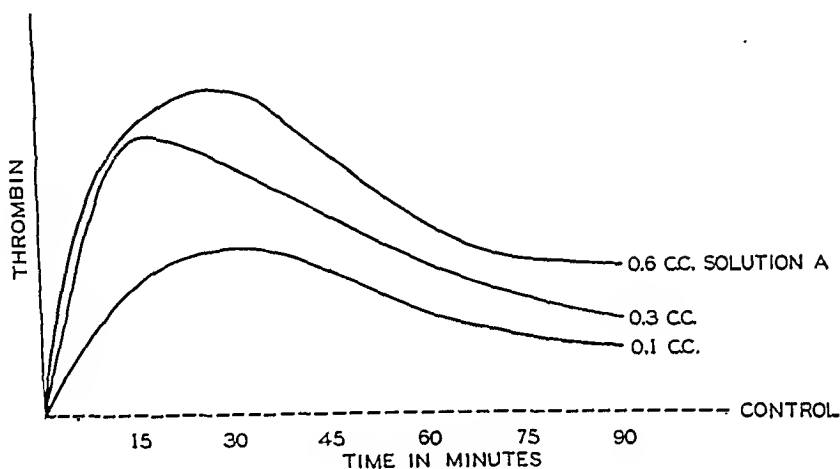


Chart 1.—Production of thrombin from prothrombin and solution A.

The same procedure which furnished solution A can be applied to a chloroform serum, which is characterized by contact of the serum and chloroform for less than twenty-four hours. In this case the solution obtained has the properties of thrombin which are more marked the shorter the time of contact with chloroform.

The isoelectric precipitation at pH 6 of diluted chloroform serum is not the only means to isolate the active fibrinolytic principle from this serum. A highly satisfactory process consists in dialyzing chloroform serum in cellophane against running tap water below 10° for three days; the precipitate of euglobulin formed is treated as the precipitate obtained by isoelectric precipitation. Both solutions, obtained either by precipitation at pH 6 or by dialysis, exhibit exactly the same properties and may, therefore, be assumed to contain the same principle.

#### DISCUSSION

The experimental data indicate that chloroform serum prepared from the blood of dogs after twenty-four hours of contact with chloroform exhibits properties different from that obtained after only one hour of contact. While chloroform serum obtained after only one hour of contact with chloroform coagulates fibrinogen with subsequent fibrinolysis, that obtained from twenty-four hours contact in the same concentration produces complete fibrinolysis

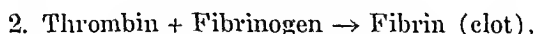
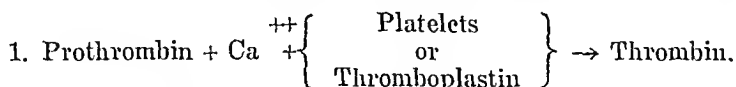
without preliminary coagulation. Very low concentration of chloroform serum obtained after twenty-four hours contact will clot fibrinogen. The clot, however, disappears very rapidly.

The saline solution of the globulin fraction derived from chloroform serum after twenty-four hours (solution A), prepared by isoelectric precipitation at pH 6, or by dialysis, differs from chloroform serum itself in several particulars. While solution A can coagulate oxalated plasma, it cannot, unlike chloroform serum, in any concentration coagulate phosphated plasma or fibrinogen to which thromboplastin has been added or pure fibrinogen itself. Prothrombin must be added to fibrinogen solutions before coagulation takes place.

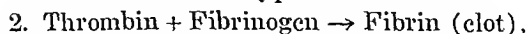
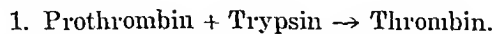
Solution A, however, does produce fibrinolysis but does so without any preliminary clotting activity. Chloroform serum does not require the presence of added prothrombin because it presumably already contains some of this substance, in either free or combined form. In certain concentrations, therefore, chloroform serum acts like thrombin, since it can produce coagulation of pure fibrinogen.

With respect to blood coagulation, solution A has enzymelike characteristics and resembles much in detail the action of trypsin reported by Eagle and Harris.<sup>3</sup> So marked was this resemblance that one wonders whether this enzymelike material could be of pancreatic origin. Pancreatectomized dogs, the pancreatectomy being confirmed by post mortem and microscopic verification,<sup>22</sup> from whose blood chloroform serum was made at intervals over several months, disproved this hypothesis. The characteristics of the chloroform serum remained unchanged.

Eagle and Harris accept the two-stage theory of blood coagulation whereby:



From their studies on trypsin they propose an enzymatic modification of this reaction as follows:



From this substitution the conclusion must be drawn that trypsin replaces calcium, platelets, and thromboplastin in the blood coagulation reaction.

The possibility that trypsin, a substance not yet identified with any blood constituent, having any connection with blood coagulation, seemed remote. The evidence that solution A resembles trypsin in many of its reactions is important, since solution A is obviously of plasma origin, requiring only the addition of chloroform to oxalated recalcified plasma to activate it.

However, the experimental evidence presented in this paper does not permit the identification of solution A with any of the substances in the coagulation reaction presented by Eagle and Harris. It contains neither thromboplastin nor fibrinogen, and its calcium ion content, if any, must be infinitesimal. Certainly

no preformed thromboplastin was present since the parent plasmas showed no production of fibrin even after standing for eight days. Neither can solution A be said to be thrombin since it does not in pure form clot fibrinogen.

If it can be said that trypsin, a substance foreign to the blood, can replace calcium plus platelets or thromboplastin in the blood coagulation reaction, it would logically follow from the experimental data, and from the fact that solution A is a true blood derivative that it too can replace the calcium thromboplastin platelet system.

Solution A is of plasma origin and can produce thrombin from prothrombin. It is highly probable, therefore, that it plays an essential role in the blood coagulation reaction. Its role is of such a nature that it would appear that calcium thromboplastin or platelets are no longer necessary in the coagulation reaction or play only a minor role.

One may go even farther than this and speculate whether the theoretical outline of blood coagulation outlined above can any longer be true, since it obviously does not lend itself to the inclusion of the existence of an enzyme theory which the evidence of this communication would appear to indicate.

The evidence for the enzymelike character of this plasma derivative may be briefly summarized. It lyzes fibrinogen, except in the presence of prothrombin, when coagulation intervenes followed by fibrinolysis. Neither calcium nor thromboplastin plays any direct role in this reaction. Thromboplastin can play a secondary role, as shown by the fact that the addition of *chloroform serum* in an amount that would normally lyze fibrinogen without coagulation, may be made to coagulate the fibrinogen if thromboplastin is added. However, thromboplastin does not have even this effect on *solution A*. It can augment the effect of prothrombin in this regard but alone has no effect on *solution A*.

As remarked earlier in the discussion, chloroform serum prepared after one hour's contact with chloroform differs in essential characteristics from that prepared after contact for twenty-four hours. A discussion of the reasons for the difference seems appropriate.

During the initial shaking with chloroform, thrombin is immediately formed and in large concentration, as shown in Table I. Fibrin is also precipitated. As time goes on the fibrin undergoes fibrinolysis due to the apparent proteolytic properties now present in chloroform serum. At the end of twenty-four hours, at which time the chloroform is removed by centrifuging and aeration, there is no longer any fibrin present.

These facts are interpreted by reasoning that under the influence of chloroform an enzymelike substance is produced which forms thrombin from prothrombin in the presence of calcium ion. The result of this reaction is fibrin formation. During the subsequent twenty-four hours the enzymelike substance proteolyzes both prothrombin, fibrinogen, if any is still present, and fibrin. Table VIII and Chart 1 clearly indicate that the enzymelike material is able to produce both of these effects. With the disappearance of prothrombin the enzyme has no longer any ability to coagulate fibrinogen, but becomes purely fibrinolytic unless prothrombin is subsequently restored. The last traces of prothrombin are removed when solution A is formed by isoelectric precipitation or dialysis of the chloroform serum.

The possibility that thrombin itself may contain as part of that complex the enzymelike material described in this paper cannot be excluded by the experimental data here presented. The failure of certain thrombin preparations<sup>21</sup> to display any such fibrinolytic activity would then be explained by the presence of thromboplastin as an impurity in such preparations. The data show that thromboplastin when present can inhibit fibrinolysis. The fibrinolytic properties of thrombin previously described by Nolf<sup>13</sup> and ascribed to the presence of one of its constituent substances thrombozyme may indicate a possible identity between thrombozyme and solution A.

The fact that the enzymelike substance contained in chloroform serum is precipitated at pH 6 and by dialysis suggests the possibility of a similarity between the active principle of solution A and the plasma substance acting on hemophilic blood, investigated by Patek, Stetson, and Taylor.<sup>15-17</sup> Should this be the case, hemophilia would be due to a lack of proteolytic fibrinolytic-clotting enzyme in the plasma.

#### SUMMARY

1. A method is devised for obtaining from oxalated plasma a proteolytic enzyme active in blood coagulation.

2. Similar to trypsin, this enzyme participates in the mechanism of blood coagulation by changing prothrombin into thrombin without calcium, thromboplastin, or platelets.

3. The proteolytic action of the enzyme on fibrinogen is counteracted by prothrombin. The action of prothrombin is reinforced in this respect by thromboplastin. Phosphated plasma and thromboplastin, without prothrombin, have no antilytic power. Prothrombin permits the enzyme to manifest its action by a clotting effect.

4. The antilytic action of thromboplastin explains why the proteolytic properties of thrombin were not found by many workers since their thrombin was prepared by means of thromboplastin.

5. The fact that the enzyme is extracted from plasma and that it reacts with prothrombin to form thrombin without intervention of calcium or thromboplastin is good evidence that the enzyme plays a primary role in the mechanism of normal blood coagulation. This role is discussed.

6. It is suggested that this enzyme is the substance, the absence of which causes hemophilia.

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## THE EFFECTS OF POLYVINYL ALCOHOL INJECTIONS ON BLOOD AND TISSUES\*

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POLYVINYL alcohol is a synthetic chemical.† Its colloidal nature, water solubility, and inertness suggested that it may prove valuable as a drug for increasing blood colloids and thus osmotic pressure and blood volume. Lowered oncotic pressure due to loss of plasma protein plays a large part in the production of nephrotic edema. Such substances as serum transfusion and intravenous gum acacia solution have been used successfully to counteract this lowered oncotic pressure. Effects of intravenous acacia were studied in this laboratory.<sup>1</sup>

Hueper,<sup>2</sup> working with rats and rabbits, reports the pathology produced by oral and intravenous administration of repeated small doses of polyvinyl alcohol. This paper reports the results of a single massive dose of polyvinyl alcohol on kidney tissue and the blood of normal dogs.

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†Developed by DuPont Company.

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Polyvinyl alcohol used in this work technically referred to as "RH-391 type B." A 5 per cent solution of this chemical in physiologic saline, warmed to body temperature, was administered to normal dogs intravenously, in one massive dose of 1 Gm. of the chemical per kilogram of body weight. Blood samples were taken before and at definite time intervals after the injection. Sedimentation rate (*Winthrop method*), cell volume, red and white blood cell counts, oxygen and carbon dioxide content, and oxygen capacity were determined on these blood samples. Blood and plasma sinears were tested for the presence of polyvinyl alcohol by *Lugol's solution* as described by Hueper. Washed cells were also tested for the chemical before and after hemolysis and urine was also tested for the presence of the chemical. Polyvinyl alcohol particles stain blue with *Lugol's solution*. Animals were observed for clinical symptoms of any ill effects, and the tissues of sacrificed animals were grossly and microscopically examined for damage caused by this chemical.

TABLE I

BLOOD DETERMINATIONS ON A HEALTHY DOG BEFORE AND AFTER INTRAVENOUS INJECTIONS OF POLYVINYL ALCOHOL\*

|   | BEFORE<br>IN-<br>JECTION | AFTER INJECTION |       |       |        |        |
|---|--------------------------|-----------------|-------|-------|--------|--------|
|   |                          | ½ HR.           | 1 HR. | 4 HR. | 24 HR. | 7 DAYS |
| Hemoglobin (per cent)                     | 127                      | 82              | 87    | 110   | 119    | 112    |
| White blood cells (thousands)             | 14.6                     | 5.6             | 5.8   | 12.9  | 19.4   | 9.4    |
| Red blood cells (millions)                | 6.8                      | 5.2             | 4.9   | 5.6   | 6.3    | 6.0    |
| Cell volume (per cent)                    | 71.9                     | 41.4            | 42.5  | 45.8  | 44.7   | 59.9   |
| Sedimentation rate, millimeters in        |                          |                 |       |       |        |        |
| 15 minutes                                | 0                        | 53              | 40    | 43    | 5      | 3      |
| 30 minutes                                | 0                        | 57              | 53    | 50    | 14     | 3      |
| 45 minutes                                | 1                        | 58              | 55    | 53    | 40     | 31     |
| 60 minutes                                | 1                        | 60              | 56    | 53    | 43     | 37     |
| CO <sub>2</sub> content (volume per cent) | 53.2                     | 54.1            | 52.2  | 55.1  | 40.9   | 46.6   |
| O <sub>2</sub> content (volume per cent)  | 20.2                     | 12.2            | 13.5  | 13.5  | 18.5   | 18.5   |

\*A single dose of 1 Gm. per kilogram of body weight as 5 per cent solution in physiologic saline.

TABLE II

BLOOD DETERMINATION ON HEALTHY DOGS AFTER INJECTION OF POLYVINYL ALCOHOL  
(Averages of all experiments)

|   | DAYS AFTER INJECTION                       |      |      |      |      |
|---|--|------|------|------|------|
|   | 7  | 14   | 20   | 30   | 45   |
| Hemoglobin (per cent)                     | 100  | 115  | 110  | 100  | 120  |
| White blood cells (thousands)             | 15.0                                       | 10.5 | 11.5 | 12.9 | 12.0 |
| Red blood cells (millions)                | 6.2  | 5.9  | 7.3  | 6.6  | 6.6  |
| Cell volume (per cent)                    | 43.6                                       | 46.3 | 46.3 | 45.3 | 47.9 |
| CO <sub>2</sub> content (volume per cent) | 52.5                                       | 51.5 | 52.3 | 46.6 | 50.4 |
| O <sub>2</sub> content                    | 14.3                                       | 15.5 | 14.9 | 14.6 | 16.3 |
| Sedimentation rate.                       | Stays increased even after forty-five days |      |      |      |      |

Eight dogs were used in these experiments. Injections were repeated on two dogs a second time. One was given a second dose of 0.5 Gm. of the chemical per kilogram of body weight twenty-one days after the first injection, and the other was given 1.0 Gm. per kilogram sixty days after the first injection. As the results of all the experiments show a similar trend, observations on only one

dog are recorded in the first table, and averages of all observations are given in the second.

Blood samples taken half an hour after the injection of polyvinyl alcohol show a marked decrease in all blood values, a dilution effect. The drop in the white blood cell count is relatively greater than the drop in other blood constituents. Unlike other blood values, the white blood cell count rises above normal after the initial drop and then comes back to normal gradually. Rise and

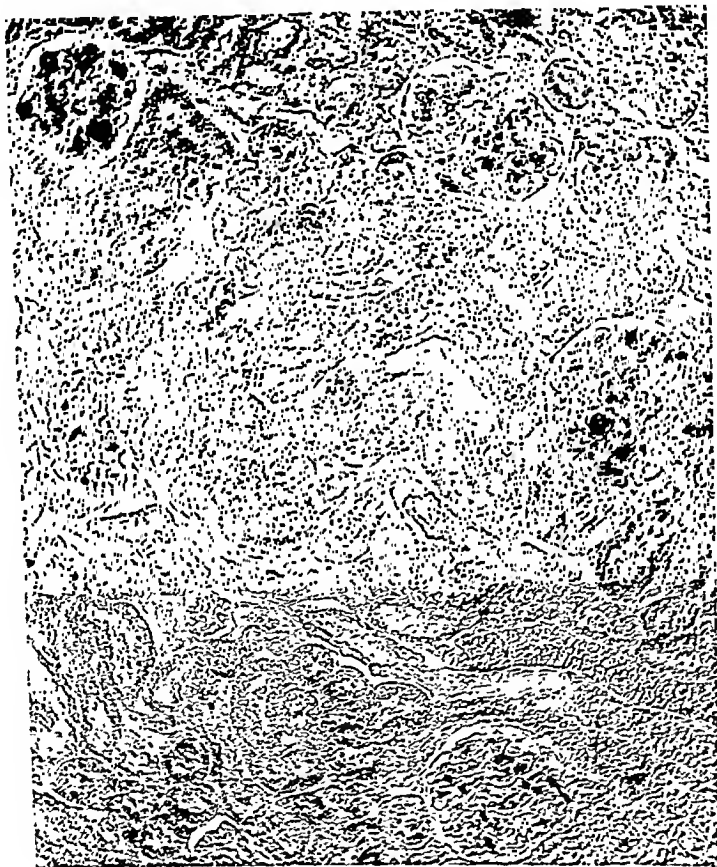


Fig. 1.—Early sections.

fall in hemoglobin, red blood cells, cell volume, and oxygen content values follow each other very closely. Carbon dioxide content of the blood falls only after twenty-four hours after the injection. Sedimentation rate is very rapid half an hour after the injection and does not even approach normal at the end of forty-five days after the injection. All other blood values return to, or approach, normal within one to three weeks after the injection. Oxygen-combining power of the hemoglobin as determined by oxygen capacity per unit of hemoglobin is undiminished.

Staining tests show that polyvinyl alcohol can be traced in the blood for forty to fifty days, and gives very strong and unmistakable reaction up to thirty

days after its injection. This chemical circulates in the plasma and does not enter the blood cells. It is excreted in urine, but to a very small degree. It accumulates in the liver, spleen, and kidney.

One dog became ill, refused to eat, vomited, developed diarrhea and a cold, lost weight, and died two weeks after the injection. The second dog lost his appetite and began to lose weight shortly after the injection, and was sacrificed. The remainder of the dogs showed an initial loss of appetite and weight, but returned to normal in a few days and remained so for the period of observation. The second injections were tolerated as well as the first.

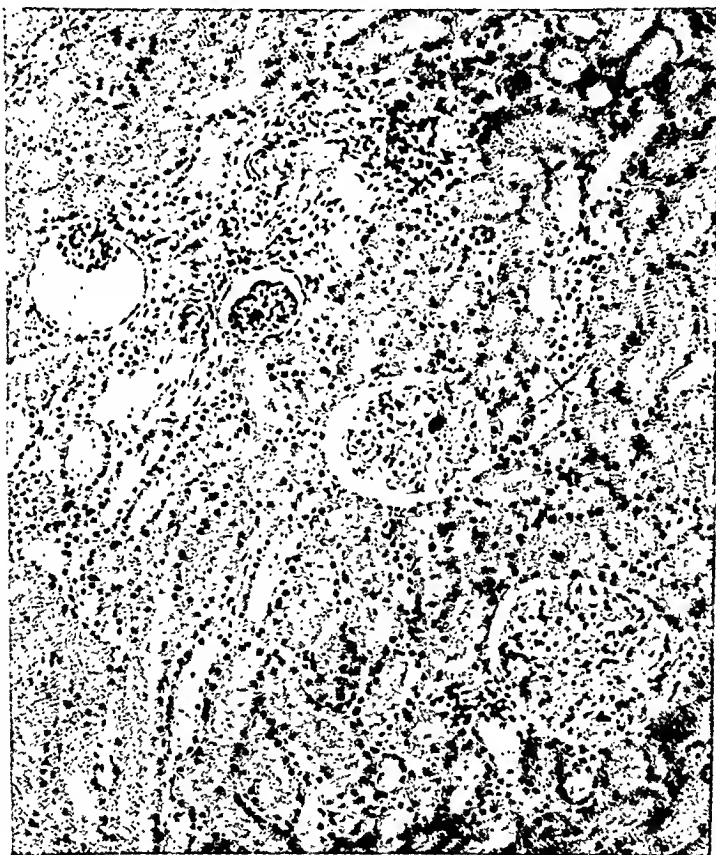


Fig. 2.—After second dose.

Tissue examination showed rather marked tubular degeneration, damage to glomerular tufts, and endothelial swelling filled with polyvinyl alcohol (Fig. 1). The tubular lumina and epithelial lining also contained the chemical. Liver and spleen tissue also showed accumulation of polyvinyl alcohol. In the tissues of the dogs given second injections, many glomerular tufts were only one-quarter normal size due to fibrosis and round-cell infiltration (Fig. 2). Hueper, working with rats and rabbits, found similar damage to tissues after several subcutaneous or intravenous injections of polyvinyl alcohol in small doses.

Although polyvinyl alcohol is an inert chemical, and animals receiving it even in massive doses seem to be normal after initial loss of weight and appetite, blood and tissue examinations show that the animal body is unable to break up and eliminate this compound, thus causing its accumulation in tissues and tissue damage, the kidney in particular. This makes it unsuitable and probably dangerous as a therapeutic agent.

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### THE TREATMENT OF ARTHRITIS BY ELECTROPYREXIA INCLUDING SOME PHYSIOLOGIC STUDIES DURING FEVER THERAPY<sup>2</sup>

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NUMEROUS reports have appeared since our original communication<sup>1,2</sup> evaluating artificial fever in the treatment of chronic arthritis. Marked differences of opinion exist as to the merits of this therapy.<sup>3-11</sup> Our study gives the results obtained with electropyrexia in 27 patients suffering from chronic infectious arthritis.

#### METHODS

The patients ranged in age from 17 to 68 years and were selected from the arthritis clinic of Northwestern University Medical School. Each patient was examined in the general medical clinic, and was followed by a re-examination in the arthritis clinic according to a definite routine. This ruled out many vague syndromes simulating arthritis, and at the same time gave us an opportunity to determine the patients' probable ability to withstand the treatment. Electropyrexia was advised after the routine measures of management had been tried without any improvement.

The treatment, given once a week, consisted of four hours of fever between 104° F. and 104.6° F. An effort was made to keep the temperature as close to 104° F. as possible. Each patient received from six to eight treatments.

At the time of the physical examination of the patients, a quantitative chemical analysis for total and fractional proteins was made, in addition to the usual laboratory test.

The patient's temperature was elevated by using a fever cabinet in combination with an inductotherm. Rectal temperature was read with an indicating

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pyrometer and recorded every fifteen minutes, together with the pulse rate and respiration. Fluids were administered to the patients in the form of 0.4 per cent sodium chloride solution. The fluid intake was limited to 250 c.c. for each fifteen-minute period unless more could be given without causing distress. In this way nausea or vomiting was seldom encountered.

None of our patients was given sedatives.

At the beginning of each treatment when the patient's rectal temperature reached 104° F., when this temperature had been maintained for four hours, and, finally, when the patient's rectal temperature had returned to normal the following physiologic tests were undertaken:

1. Blood pH determinations by means of the Dole glass electrode were made on 10 patients.
2. Blood density determinations, using the Barbour and Hamilton<sup>12</sup> falling-drop method, were made on 11 patients.
3. Ascorbic acid concentration of the blood was determined in 18 patients by the method of Farmer and Abt.<sup>13</sup>
4. Circulatory changes were measured in the ring finger of the left hand by means of the Johnson air conduction plethysmograph on 12 patients.
5. Blood pressure was recorded before and after each treatment for 10 patients.

TABLE I

|                     |                  |
|---------------------|------------------|
| Markedly improved   | 6 patients (22%) |
| Moderately improved | 6 patients (22%) |
| Slightly improved   | 9 patients (33%) |
| Unimproved          | 6 patients (22%) |
|                     | 27               |

## RESULTS

Following fever therapy, the patients returned to the arthritis clinic where the clinicians of that department evaluated the improvement. The results are tabulated in Table I. The criteria used in evaluating improvement in this series included the patient's statement of relief of pain and stiffness. The degree of fatigue was less, and its occurrence was not so frequent. The objective evidence consisted of decreased swelling, increased motion of joints without pain, and gain in weight. Improvement was graded according to the period of time relief persisted.

Slight improvement consisted of temporary improvement, with decreased pain and stiffness in joints for a variable period of time. Moderate improvement consisted of decreased pain and stiffness, decreased swelling, and increased motion in the joints for three months or longer. Marked improvement consisted of decreased pain, stiffness and swelling, with marked increase in joint motion; patient able to return to work. The period of relief lasted for one year or longer.

It will be noted (Table I) that in only six (22 per cent) of the group were the results sufficiently striking over a period of one year or longer to be classified as markedly improved. In no case in the group was a complete permanent result obtained so that the designation "cured" could be used.

TABLE II  
LABORATORY DATA  
A. Specific Gravity of Blood

|   | ELAPSED<br>TIME<br>HOURS | AVERAGE<br>VALUE | 2 $\sigma$ DIFFER-<br>ENCE $\pm$ | CHANGE $\pm$<br>FROM<br>CONTROL | NO. OF<br>OBSERVA-<br>TIONS |
|---|--------------------------|------------------|----------------------------------|---------------------------------|-----------------------------|
| Control                                     | 0                        | 1.0536           | -                                | -                               | 30                          |
| Temperature reached<br>104° F.              | 2                        | 1.0555           | 0.0018                           | 0.0019                          |                             |
| Temperature four hours at<br>104°-104.6° F. | 6                        | 1.0552           | 0.0018                           | 0.0016                          |                             |
| Temperature normal                          | 8                        | 1.0514           | 0.002                            | 0.0008                          |                             |

B. Ascorbic Acid Blood Concentration  
mg./100 c.c.

|   |   |      |       |      |    |
|---|---|------|-------|------|----|
| Control                                     | 0 | 0.62 | -     | -    | 77 |
| Temperature reached<br>104° F.              | 2 | 0.68 | 0.104 | 0.06 |    |
| Temperature four hours at<br>104°-104.6° F. | 6 | 0.66 | 0.106 | 0.04 |    |
| Temperature normal                          | 8 | 0.64 | 0.088 | 0.02 |    |

C. Blood pH

|   |   |       |        |       |    |
|---|---|-------|--------|-------|----|
| Control                                     | 0 | 7.41  | -      | -     | 33 |
| Temperature reached<br>104° F.              | 2 | 7.55  | 0.038  | 0.14  |    |
| Temperature four hours at<br>104°-104.6° F. | 6 | 7.52  | 0.0396 | 0.11  |    |
| Temperature normal                          | 8 | 7.418 | 0.028  | 0.003 |    |

D. Blood Pressure Changes

|                       | AVERAGES    |            | DIFFERENCE | 2 $\sigma$ DIFFER-<br>ENCE $\pm$ |  | OBSERVA-<br>TIONS |
|-----------------------|-------------|------------|------------|----------------------------------|--|-------------------|
|                       | BE-<br>FORE | AFT-<br>ER |            |                                  |  |                   |
| A. Systolic pressure  | 129         | 108        | -21        | 7                                |  | 24                |
| B. Diastolic pressure | 83          | 69         | -14        | 6.4                              |  |                   |
| C. Pulse pressure     | 46          | 39         | -7         | -                                |  |                   |

In Table II data of the laboratory studies are given. The average blood pH for the group was 7.415 for the control period, 7.55 at a temperature of 104° F., 7.52 four hours later, and 7.418 when the temperature dropped to normal. This shows an uncompensated alkalosis during the febrile period.

The greatest change in the blood density occurred during the induction of the fever, but inasmuch as the only significant figure was in the fourth decimal place, we have considered the change as insignificant.

Fig. 1 shows the typical finger volume changes of the average patient secured by means of the plethysmograph. The photographic record, Fig. 1, clearly shows the magnitude of the changes found. The maximum response occurred between a temperature of 103° F. and 104° F. Occasionally the maximum response was recorded at 102° F.

No significant changes in the ascorbic acid concentration of the blood plasma were found which could be attributed to the influence of electropyrexia.

The average of 26 determinations of total serum protein, albumin, and serum globulin of the blood prior to treatment are shown in Table III. The total serum protein of the blood ranged from 6.02 per cent to 9.2 per cent. The great majority (18) were within the accepted standard range of 6.0 to 8.0 Gm. per 100 c.c. Eight patients ranged from 8.1 to 9.2 per cent. Three patients showed a higher percentage of serum albumin than the normal range of 3.6 to 5.4 per cent. The serum globulin was lower than the normal range of 1.5 to 3.4 in four patients, and was higher in four. The albumin-globulin ratio was reversed in two patients. The average ratio for the 26 patients was 1.9. One patient was below the accepted normal range of 1.2 to 2.6, and six were above. The average value for fibrinogen was 0.42.

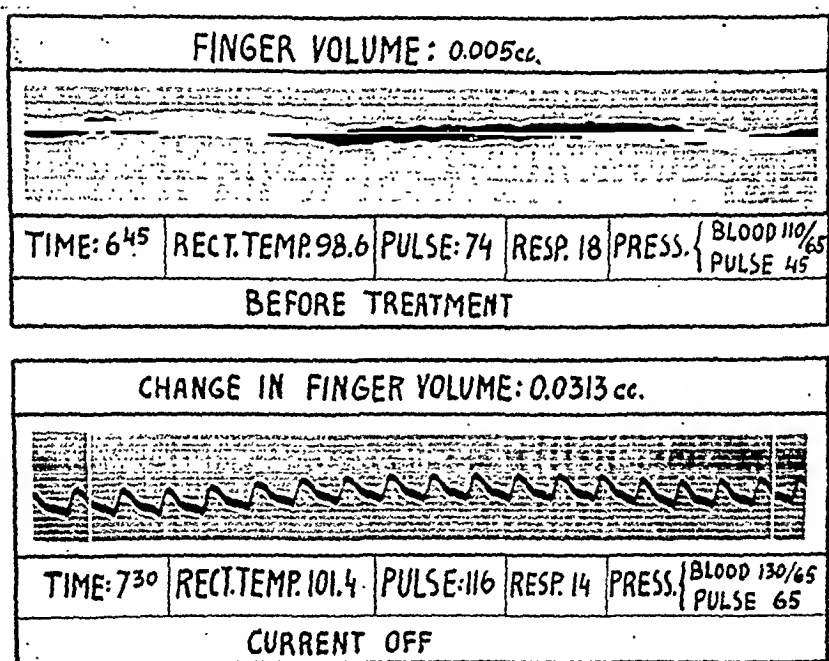


Fig. 1A.

Fig. 1A, B.—The effect of artificial fever on the blood volume of the finger as measured by the air conduction plethysmograph.

The systolic blood pressure averaged 129 mm. Hg before treatment, and the diastolic pressure averaged 83 mm. Hg. After treatment both systolic and diastolic pressures had fallen to 108 and 69 mm. Hg, respectively (Table II D). These were significant changes as can be seen by referring to Table II.

#### PATIENT REACTIONS

The majority of the patients tolerated the treatment quite well. The complaint of headache was not infrequent. Occasionally a patient had paraesthesia of both hands, and dizziness. One patient of this series vomited. An unlisted patient at the completion of his first treatment suddenly failed to respond to



questions, and then became irrational. There was a brief period of paralysis of the right arm and right leg. The left nasolabial fold was eradicated, and the mouth was drawn down on the left side. After a period of thirty minutes the patient was able to respond to questions but was still confused. Still another patient, L. B., developed an auricular fibrillation, was nauseated, had marked circumoral pallor, cold skin, and complained of faintness and abdominal pain. This instance of shock developed during the sixth and last treatment.

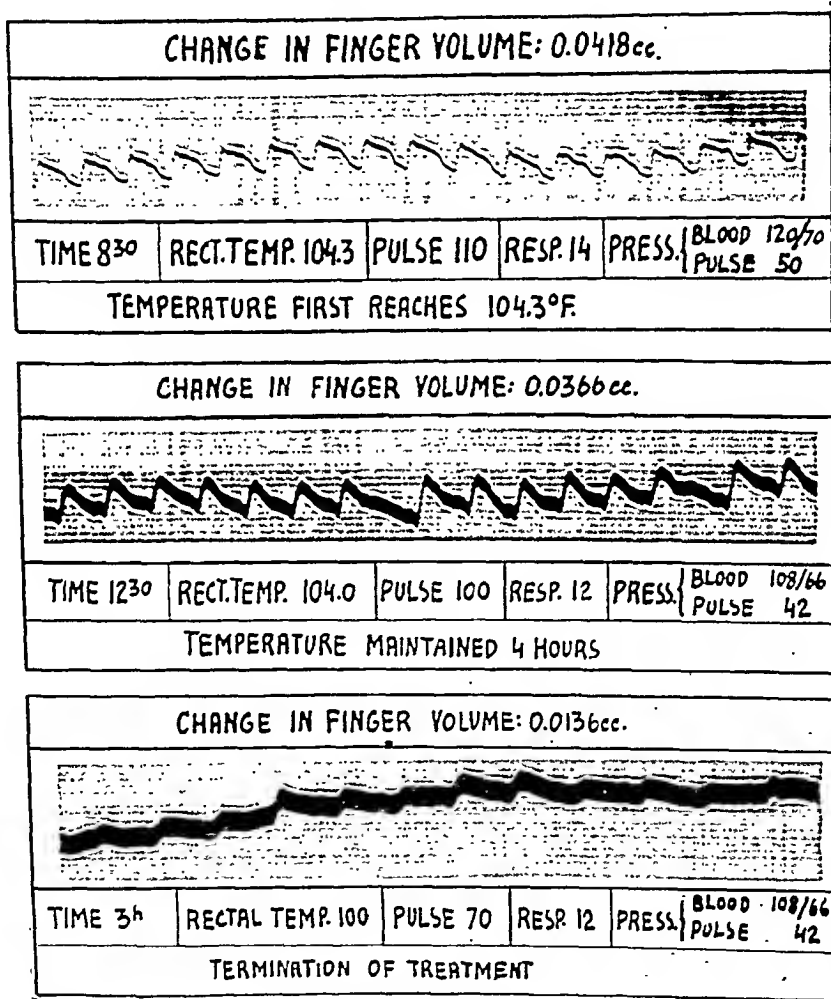


Fig. 1B.

Patient H. G. during his sixth and last treatment developed marked cyanosis as well as a twitching of the fingers of both hands. The heart tones were distant. The blood pH was 7.62. This patient's condition undoubtedly could have been relieved by the administration of carbon dioxide inhalations. Ten patients not listed completed only one or two treatments for various causes. One such patient after the second treatment developed a typical papular type of rheumatic rash, and treatment was, therefore, discontinued.

## DISCUSSION AND SUMMARY

That none of our patients was "cured" has neither surprised nor dismayed us.

The current report<sup>14</sup> of the American Rheumatic Commission recently published by Hench and his collaborators shows only too well how far we are from the solution of the problems of arthritis. It indicates that there is far from unanimity among recognized authorities as to a standard classification of the disease in its various manifestations. The multiplicity, as well as the variety of the therapeutic procedures used for its control, certainly shows that the fundamental concepts of the disease are as yet little understood. Hench<sup>10</sup> states that fever therapy may benefit arthritis by (1) direct bacteriolytic or bacteriostatic effects due to an influence of heat itself on bacteria; (2) a local effect from vasodilatation providing an augmented blood supply to inflamed tissues; (3) increasing indirectly the formation or mobilization of immune bodies; (4) a general effect from the heightened metabolism incident to fever. Whatever may be the factor that accounts for the benefit of fever therapy in arthritis, Hench is certain that selection of the patient is of prime importance. We select our cases from the atrophic group alone, preferably those who have not had their arthritis longer than two years. Those whose arthritis is well advanced and arrested can hope for only slight improvement, such as gain in weight, less pain and stiffness, less fatigue, but very little improvement in joint function. Unquestionably no one yet can claim to have solved the problem of the correct temperatures to be employed, the duration of the fever or its frequency of application, nor have the possibilities of fever therapy used in conjunction with other procedures been fully explored.

Our patients were all referred to our clinic from outside sources for treatment and in most cases long after other forms of therapy had failed to give relief.

In this study it was our aim to evaluate further the results of the short fever curve maintained at 104° F. for four hours. These additional patients tend to confirm our previous impression<sup>2</sup> that improvement of the patient is greater and of longer duration when the fever is maintained for eight hours. In this series we also gave a minimum of six treatments, but undoubtedly this is far too few. It may well be that such a series of treatments repeated once or twice a year might produce much better results.

The plethysmographic records would seem to indicate that a rectal temperature exceeding 103° F. to 104° F. is neither necessary nor desirable. The maximum changes in the peripheral circulation apparently occur at this temperature level, and if increased circulation is the therapeutic objective, then these temperatures should not be exceeded. High temperatures are contraindicated for this type of patient because many of them give evidence of a vasomotor instability, and have a low threshold for exhaustion. Furthermore, it is our opinion that they should not be subjected to high environmental temperatures. It can be seen from Table II *D* that there is a very significant drop in blood pressure as a result of the fever treatment. This may be due to a low-

ered peripheral resistance, caused by the marked vasodilatation which is present, and to a lowered blood volume level.

Investigators vary markedly in their reports as to what constitutes the normal blood levels of ascorbic acid either in health or in disease. Abt and Farmer<sup>15</sup> consider blood plasma values below 0.7 mg. per 100 c.c. to be suboptimal. Danm, Boyd, and Paul<sup>16</sup> state that the normal ascorbic acid content of the blood ranges between 0.7 and 2.0 mg. per cent. The assumption has been made that fever therapy increases vitamin C requirement, but direct experimental proof is lacking. While the average ascorbic acid concentration of the blood for the group was 0.62 mg. before treatment, the range varied from 0.28 to 0.72. Many values found in these patients are considered to be very low normals. Thirty-three of the 77 determinations before treatment showed a level below 0.5 mg. This is of interest in light of Rhinehart's investigations. He found that subacute or chronic vitamin C deficiencies in guinea pigs produced an arthropathy with manifold similarities to rheumatoid arthritis, and that superimposed infection accelerated or accentuated the pathologic processes. Infection in the presence of vitamin C nutrition, he noted, failed to produce arthritis.

TABLE III  
DATA OF BLOOD PROTEIN ANALYSIS  
(26 Determinations)

|                      | RANGE   |         | AVERAGE |
|----------------------|---------|---------|---------|
|                      | MAXIMUM | MINIMUM |         |
| Total blood proteins | 9.2     | 6.02    | 7.37    |
| Albumin              | 7.0     | 3.73    | 4.76    |
| Globulin             | 4.7     | 1.02    | 2.53    |
| Fibrinogen           | 0.82    | 0.17    | 0.42    |
| A./G. ratio          | 5.2     | 0.84    | 1.9     |

It is apparent that artificial fever produces a significant elevation of the blood pH. The average pH value of 7.55 found at a temperature of 104° F. indicates a state of uncompensated alkalosis. The loss of carbon dioxide by the body through the lungs and sweat during the fever is undoubtedly of prime importance in increasing the alkalinity of the blood. The skin is a path of carbon dioxide loss of some importance. Koehler<sup>17</sup> is of the opinion there is a direct correlation between cyanosis and certain types of alkalosis. Mild cyanosis is not an uncommon occurrence in fever therapy. Koehler believes that fever alkalosis is due to increased lung ventilation and the rapid elimination of carbon dioxide from the blood, thus causing a carbon dioxide deficit, which in turn results in the passage of sodium ions into the tissue fluids and partially into the urine. Landis and collaborators<sup>18</sup> do not believe that hyperventilation is the sole cause of the pH change, but is dependent on several factors, such as the kidneys, the degree of sweating, and lactic acid formation.

Bischoff, Ullmann, Hill, and Long<sup>19</sup> do not agree with Koehler that an anoxemia exists in the presence of a fever alkalosis. As a result of the lowered carbon dioxide tension in the blood and increased pH, the stability of the oxyhemoglobin increases. If the circulation and the metabolism did not increase at the same time, the question would be quite simple. With an increase

in circulation, however, the tissues are exposed to more blood per unit of time so that the effect of the hemoglobin stability might be offset if the demand for more oxygen due to increased metabolism were not too great. It would seem then that instead of supplying the very distressed patient with oxygen as has been the tendency during the past few years, that it might be far more effective to give 8.5 per cent of carbon dioxide or even have the patient rebreathe air from a rubber bag. The unusual symptoms described for four patients are those resulting from hyperventilation of varying degree. Recently, Hinshaw and Boothby<sup>20</sup> have described this syndrome. Recently, also, we have undertaken some rebreathing experiments using a Boothby-Lovelace-Bulbulian face mask and as a result it is our opinion that had rebreathing been instituted during the period of hyperventilation all these patients would have completed treatment successfully.

Several investigations have been made chiefly on animals, a few on human beings, but none under comparable conditions, on the effect of fever on hemoconcentration. Observed blood changes<sup>21</sup> have frequently been explained as due to a concentration phenomena. According to Howell,<sup>22</sup> the specific gravity of human blood may vary from 1.041 to 1.067, the average being 1.055. All of our determinations (Table II) came within this range. Gibson and Kopp<sup>23</sup> state that one of the major physiologic effects of artificial fever induced by physical means is a diminution in circulating blood volume. They state that the degree of reduction in plasma volume is determined by the difference in rate of outflow by skin, lungs, and kidneys, and of effective absorption of fluids administered. If insufficient fluids are given the tissues, fluids of the body are drawn upon for the maintenance of plasma volume with resultant dehydration. Undoubtedly, the blood density will be dependent upon the balance of fluid loss and fluid gain, and one might readily criticize our work in that we did not control these variables. As previously stated, we gave fluids both in regard to quantity and rate, according to our judgment of the patient's need. We were interested, primarily, in ascertaining changes in hemoconcentration under the conditions of our therapeutic regime. In the thirty determinations of the specific gravity of the blood no significant changes were found. Apparently hemoconcentration is not the mechanism involved to account for the changes occurring in the blood cell count when fluids are administered during the course of treatment.

Our analysis of the blood serum proteins does not support the work of Davis.<sup>24</sup> Davis studied the blood plasma of nine patients with rheumatoid arthritis and found an increase of the globulin and fibrinogen as well as a lowered albumin of the blood plasma. The average albumin:globulin ratio of his nine patients was 0.9, while ours was 1.9. Two of our patients showed a reversal of the usual ratio (0.84 and 0.85, respectively). In both instances the reversal was due to an increase of the globulin fraction. Block<sup>25</sup> seems to believe that the albumin:globulin ratio is shrouded in a considerable degree of empiricism. Peters<sup>26</sup> states that studies of protein disturbances without simultaneous data on salt and fluid balance are valueless.

The average values for the total serum proteins, albumin, globulin, and fibrinogen of our patients conformed very well to the average normal values.

## CONCLUSIONS

1. Twenty-seven patients suffering from chronic infections arthritis were submitted to a fever of  $104^{\circ}$  F., maintained for four hours, and induced by electromagnetic induction.

2. Various physiologic observations, such as blood pH, ascorbic acid concentration of the blood, the specific gravity of blood, finger volume changes, total and fractional blood proteins, and blood pressures are reported.

3. Twenty-two per cent of the patients were markedly improved; 22 per cent moderately improved; 33 per cent slightly improved; and 22 per cent unimproved.

4. This form of therapy is not specific for chronic infections arthritis but should be used in properly selected patients.

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## THE AMOUNT OF PLASMA AND WHOLE BLOOD IN THE LUNGS OF DOGS\*

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FEW previous studies concerning the amount of blood in the lungs are available. Stewart<sup>1</sup> calculated the quantity of blood in the lungs in dogs from the cardiac output per minute and the pulmonary circulation time, using the formula  $V = Q \frac{60}{T}$ . He expressed it as per cent of the total blood volume (estimated to be one-thirteenth of the body weight). His figures ranged from 11 to 26 per cent, and averaged 17 per cent. Kuno's<sup>2</sup> figures of 8.8 per cent to 19.44 per cent of total blood volume (estimated at one-fourteenth of body weight) in both lungs were obtained by colorimetric determination of the blood content of lungs from heart-lung preparations. Blumgart and Weiss<sup>3</sup> determined cardiac output and net pulmonary circulation time in human beings and, using Stewart's formula, calculated that the lungs contain 10 to 41 per cent of the total blood volume (estimated to be one-thirteenth of the body weight), the average being 21 per cent. Bruns<sup>4</sup> found that 6.3 per cent of the total blood volume in rabbits was in the lungs. Drinker, Churchill, and Ferry<sup>5</sup> tabulated the results of some previous investigators<sup>6-9</sup> and found, in three series of experiments, that in the rabbit the lungs contained 1/10.7 to 1/12.5 of the total blood volume at the end of inspiration and 1/15.3 to 1/17 of the total blood volume at the end of expiration. We have attempted to reinvestigate the question by a direct method of measuring the amount of plasma and (by calculation) whole blood in a single isolated lung.

### PROCEDURE AND METHOD

Large normal mongrel dogs were used. Anesthesia was induced by the intravenous administration of pentobarbital (nembutal) in doses of 32.5 mg. per kilogram of body weight. After intubation of the trachea the chest was opened through the left fourth intercostal space. Adequate pulmonary ventilation was maintained by means of an Erlanger respirator. The pericardium was opened. The left branch of the pulmonary artery and the three left pulmonary veins were isolated and encircled separately by pieces of No. 1 chromic catgut, which were brought out through glass tubes, so that tension on the catgut closed the lumina of the encircled vessels. These tubes were bent in such a way as to conform to a space between the heart and the left lung, so that neither chambers

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of the heart nor portions of the left lung would be collapsed by mechanical interference. The glass tubes protruded from the chest wall, the layers of which were closed tightly about the tubes. Just before completing the closure of the wound, the lungs were fully inflated. To prevent pneumothorax, the ends of the glass tubes which protruded from the chest wall were sealed with bone wax through which the catgut ligatures passed. A moderately stout rubber band was then fastened to each catgut ligature. In Experiment 1 the intratracheal tube was removed, and spontaneous respiration was established. In Experiments 2 and 3 intermittent insufflation was continued throughout the remainder of the experimental period. After allowing several minutes for stabilization of the

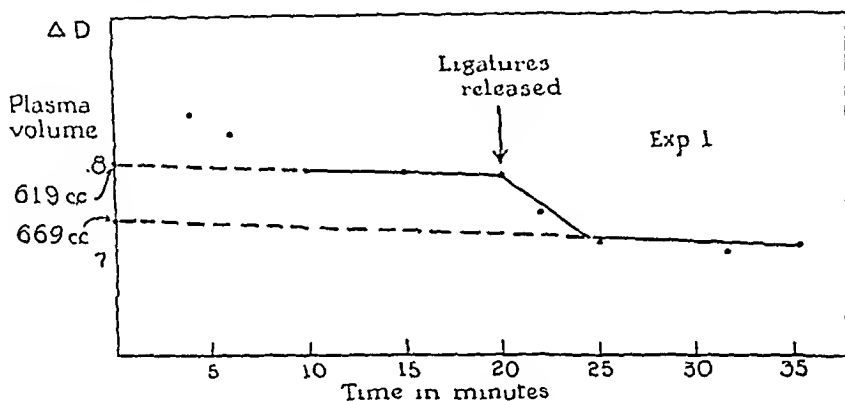


Fig. 1.—The concentration of dye in the circulating plasma is charted in relation to the intervals following (1) a single injection of dye, and (2) release of the ligatures. By extrapolation of the slopes thus obtained the circulating plasma volume before and after the release of the ligatures was determined. The difference represents the amount of plasma contained in the left lung.

circulation, the left lung was isolated from the general circulation by simultaneous traction on the four rubber bands. The plasma volume in the remainder of the circulatory system was then determined by the dye method of Gibson and Evans.<sup>10</sup> After twenty-one, twenty-one, and twenty-two minutes, respectively, in the three experiments, the ligatures were released, allowing the undyed plasma trapped in the lung to re-enter the general circulation. Blood samples for total plasma volume determinations were taken at intervals during the following twenty minutes.

The volume of plasma in the left lung was calculated as follows (see Fig. 1): The amount of circulating plasma was determined on the basis of the slope of the disappearance of the dye from the blood stream with the ligatures closing the lumina of the vessels of the left lung. On release of the ligatures the undyed plasma contained in the left lung further diluted the remainder of the plasma, and the total plasma volume was determined from the new disappearance slope (extrapolated to the time of the dye injection) obtained from the dye concentration of the blood samples taken after mixing was complete. The difference between the two volumes was taken to represent the amount of plasma contained in the left lung while it was isolated from the general circulation. Blood for hematocrit determinations was taken during both phases of the experiment.

TABLE I

CIRCULATING PLASMA VOLUME AND AVERAGE HEMATOCRIT BEFORE AND AFTER RELEASE OF THE LIGATURES; CALCULATED VOLUME OF PLASMA TRAPPED IN THE LEFT LUNG AND PERCENTAGE OF TOTAL PLASMA VOLUME IN THE LEFT LUNG AND IN BOTH LUNGS

|  | PLASMA<br>VOLUME<br>(C.C.) | AVERAGE<br>HEMATO-<br>CRIT | PLASMA<br>VOLUME IN<br>LEFT LUNG<br>(%) | PLASMA IN<br>BOTH<br>LUNGS<br>(%) | REMARKS   |
|--|----------------------------|----------------------------|---|-----------------------------------|---|
| Exp. 1, 19 kg.<br>Ligatures closed<br>Ligatures open<br>Volume of plasma in<br>left lung | 619<br>669<br>50           | 50.5<br>50.25              | 7.4                                     | 18.5                              | Animal breathing spontaneously<br>No pneumothorax |
| Exp. 2, 27 kg.<br>Ligatures closed<br>Ligatures open<br>Volume of plasma in<br>left lung | 910<br>962<br>52           | 58.4<br>59.7               | 5.4                                     | 13.5                              | Intermittent inflation<br>Pneumothorax developed  |
| Exp. 3, 27 kg.<br>Ligatures closed<br>Ligatures open<br>Volume of plasma in<br>left lung | 920<br>995<br>75           | 57.2<br>60.1               | 7.5                                     | 18.7                              | Intermittent inflation<br>No pneumothorax         |

## RESULTS

The results are shown in Table I. The left lung contained 7.4, 5.4, and 7.5 per cent of the plasma volume, respectively, in these three experiments. It has been shown that in dogs, the quantities of blood in the right and left lungs are in the proportion of 3:2.<sup>3, 4</sup> On this basis, the portion of the plasma volume in both lungs in these three animals was estimated to be 18.5, 13.5, and 18.7 per cent. The results in Experiments 1 and 3 are in close agreement, but the lower value in Experiment 2 may have been due to a pneumothorax of undetermined extent which was detected at the end of the experiment, as it has been shown that the quantity of blood in a lung decreases with collapse of lung tissue.<sup>4, 11</sup> Hematocrit values are shown in relation to closure and release of the ligatures in Fig. 2. There was little change throughout the procedure in Experiment 1, but in the other experiments there was a marked tendency for the hematocrit to rise for some time after the release of the ligatures. The possible significance of these findings is considered below.

## DISCUSSION

The validity of the results obtained depends upon several factors affecting the accuracy of the method of plasma volume determination employed and upon the physiologic effects of the experimental procedures upon the circulation.

The accuracy of the plasma volume determinations by dye methods depends upon the complete mixing of the dye and plasma, and this, in turn, depends upon maintaining an adequate circulation as to both volume and speed. It has been demonstrated that under similar experimental conditions the sudden occlusion of one branch of the pulmonary artery does not cause a significant change in the cardiac output.<sup>12</sup> Thus, the same amount of blood goes through one lung as formerly went through both lungs, so that the circulation time is



decreased. Mixing of the dye and plasma was, therefore, thought to be satisfactory. The reliability of the so-called "indirect" method of determining changes in plasma volume with a single dye injection depends upon (1) the constancy of the intrinsic color (or optical density at a given wave length) of the plasma itself, and (2) the accuracy with which sudden changes in plasma volume are reflected by the differences in the disappearance slopes obtained. In the former instance, if there is marked blood concentration or dilution due to shifts in protein and pigment-free fluid, the optical density of the serum itself

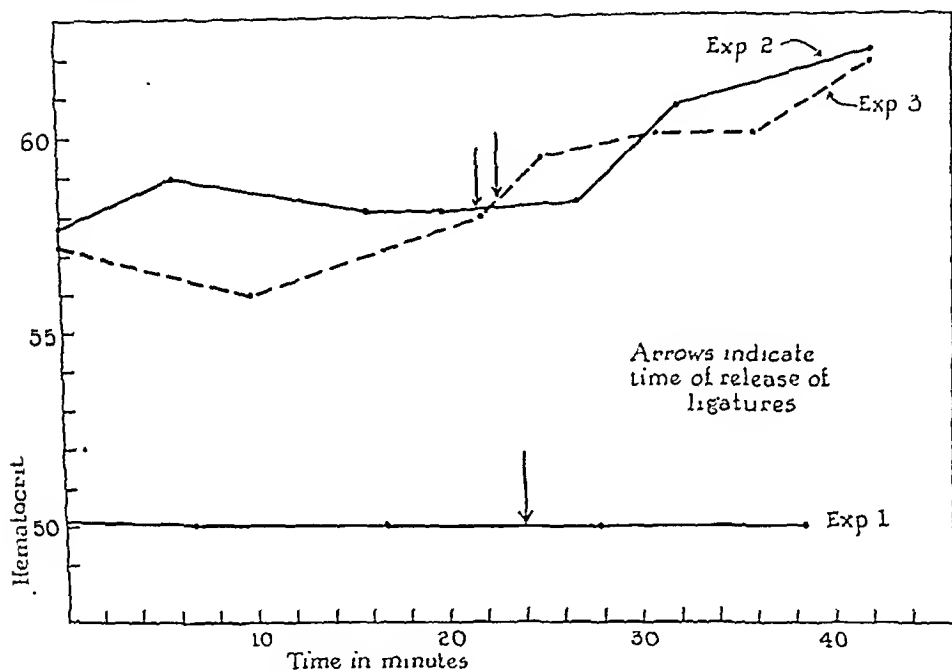


Fig. 2.—The hematocrit values in relation to the release of the ligatures.

will increase or decrease, and the net optical density, presumably due solely to the dye present, will give a distorted value. The magnitude of this error varies inversely with the concentration of dye used. Since the density value of dye in these experiments was from ten to twenty times that of dye-free serum, this source of error can be disregarded. There is little doubt that the extrapolation of a disappearance slope of the dye affords the most accurate dilution value upon which to determine the plasma volume. But if during such a control period, any great quantity of the dye disappears from the circulation, then the volume calculation based upon a second slope obtained after a sudden dilution, based on the same colorimetric constant for the original amount of dye injected, will be in error. In these experiments, not over 2 per cent of the dye left the blood stream during the time the initial slope was being obtained. Also, not over 1 c.c. (or about 1 per cent of average plasma volume) was withdrawn in any one. Therefore, this objection may be dismissed as insignificant.

Far more serious criticisms may arise on the basis of the physiological change produced by the experimental procedure itself. It has been demonstrated that

no communication exists between the bronchial and pulmonary arteries, but there is a communication between bronchial arteries and pulmonary veins.<sup>13</sup> This communication is by means of capillaries, and the differences in size between the bronchial and pulmonary vessels do not suggest the possibility in these experiments of any great degree of mixture between dye-stained blood in the systemic circulation and dye-free blood in the pulmonary vessels of the left lung. However, such a communication might permit a small quantity of whole blood to enter the pulmonary circulation from the systemic circulation, but this quantity of blood would be added to that already isolated in the lung, there being no normal avenue by which blood may escape when once it is trapped in a lung by occlusion of the pulmonary veins. The disappearance of dye from the circulating blood stream through the bronchial arteries would not affect the determination of circulating plasma volume, because both dye and plasma would leave the systemic circulation in such proportions as to be without effect upon the concentration of dye in the systemic circulation. Similarly, when the tourniquets were released, the dilution which followed would be due to the original amount of dye-free plasma trapped in the lung.

Some anoxia may have occurred in the isolated lung, and the resultant increased permeability of the parenchymal capillaries may have permitted an escape of plasma, or of fluid poor in protein (and hence, dye) from the vascular bed. The rise in hematocrit in Experiments 2 and 3 (Fig. 2), after the release of the ligatures, suggests that this may have occurred. However, if the rise in hematocrit of the peripheral blood were due entirely to its mixture with the blood trapped in the left lung, the hemoconcentration should have been limited to the period in which dilution of dye occurred and should not have persisted thereafter (Fig. 1).

If stagnation of blood in capillaries in the dependent portions of the left lung occurred during the period in which it was isolated by the ligatures, it is possible that all the blood trapped in the lung was not carried out when the circulation was re-established. This possibility cannot be ruled out, but in that event the values for the amount of plasma contained in the lung would be too low.

We believe these results represent with reasonable accuracy the amount of plasma contained in the lungs of the dog in the resting state. No attempt has been made to determine the total blood volume in the lung from the change in plasma volume and the corresponding hematocrit values. It is not known definitely whether the ratio of plasma to cells is the same throughout the circulation, but there is increasing evidence that the corpuscular concentration is much less in capillaries than in large central or peripheral vessels, and that total blood volume calculated from the plasma volume and the hematocrit of blood from a large peripheral vessel are too high. It seems probable, however, that the hematocrit values of blood from the jugular vein (source of blood samples for hematocrit here) should not differ greatly from that in the pulmonary artery or veins, and it seems fair to say that the percentage of total blood volume in the lung is roughly equivalent to the percentage of plasma volume in the lung.

The results of this short series of experiments would not in themselves be of great significance, but there is close agreement between two of the determinations (Experiments 1 and 3), and it is believed that the presence of a pneumothorax accounts for the difference in the third (Experiment 2). The average value (17 per cent) is higher than that of Plumier (10 per cent), but compares favorably with that of Stewart (17 per cent). Our results in animals cannot be assumed to apply to human beings, yet it is interesting to note that Blumgart and Weiss<sup>3</sup> found that in man an average of 21 per cent of the total blood volume was contained in both lungs.

#### SUMMARY

The quantity of blood contained in the left lung was trapped by the simultaneous occlusion of the vessels to that lung while both lungs were fully expanded. From determinations of the circulating plasma volume, during this period and after the release of the occlusion, the quantity of plasma trapped in the left lung was measured.

In three experiments, 7.4, 5.4, and 7.5 per cent (average 6.8 per cent) of the plasma volume was contained in the left lung. On the basis that, in the dog, the right lung contains one and one-half times as much blood as the left lung, the quantity of plasma in both lungs averaged 17 per cent. For reasons explained, a similar percentage of total blood volume may be assumed to be contained in the lungs.

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## IODINE ALLERGY\*

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THE widespread use of iodine and its salts in modern diagnostic and therapeutic techniques makes the finding of unusual reactions to these drugs extremely likely. Although the use of tincture of iodine as an antiseptic has fallen off in recent years, the use of the iodides, organic and inorganic, has increased because of their value in diagnosis. Prolonged intake of iodine or its salts gives rise to cutaneous eruptions more often than does the continued intake of any other single drug. However, these cases have to be distinguished from others that react in an unusual way to only a minute quantity of iodine. The latter patients are said to exhibit iodine hypersensitivity or allergy.

A patch test made with a drop of tincture of iodine, and read twenty-four hours later revealed marked redness and swelling over the site in all the hypersensitive patients. If this can be shown to be a general finding in other cases, it can be used as a diagnostic test. Other nonsensitive persons tested in this way did not show this reaction.

### CASE REPORTS

CASE 1.—Reaction following the drinking of a saline laxative containing a minute quantity of iodine.

G. B., an 18-year-old girl, took a teaspoonful of an imported Italian salt, which was found to contain a very small quantity of iodine. Twelve hours later her eyes became red, her face started to swell, and she developed a pin-point, reddened eruption over her entire body, closely resembling that of scarlet fever. Her temperature was normal. An iodine patch test was markedly positive, and the qualitative examination of the urine for iodine and iodides was negative. The white blood cell count and the differential were within normal limits. The rash cleared rapidly after the administration of 10 grain capsules of sodium chloride every two hours for six doses. This patient had no previous history of sensitivity to iodine in any of its forms. She had used the salt repeatedly for years, but only occasionally during the preceding year.

CASE 2.—Reaction following the taking of a commonly used throat lozenge containing anesthesin and calcium iodide.

R. F., an 18-year-old girl, took a lozenge for relief of sore throat. Twelve hours later she developed a slight fever, swollen neck glands, slight edema of the face, and a generalized, reddened pin-point cutaneous eruption resembling that of scarlet fever. Her throat was slightly red; differentiation from scarlet fever in this case was difficult. However, the Schultz-Carlton test was negative, and an iodine patch test was markedly positive. The white blood cell count was 9,200, with 84 per cent polymorphonuclear cells. The qualitative

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examination of the urine was negative for iodine and iodides. The symptoms cleared up in twenty-four hours after the taking of several fruit drinks containing large quantities of ordinary table salt, and without other medication.

After careful questioning this girl gave a history of having had a similar eruption many years before, which was diagnosed as scarlet fever. She had no sore throat at the time. She recollected having bruised her knee and applying tincture of iodine. The bruise turned a livid red, and a generalized rash appeared approximately twelve hours later.

**CASE 3.**—Cutaneous reaction following the insertion of a small piece of iodoform gauze after removal of a lipoma of the neck.

P. S., a 45-year-old male, had a lipoma removed under general anesthesia. A small amount of iodoform gauze was placed in the wound as a drain. Several hours later his temperature rose to 102° F. He showed lassitude, refused to take food, and had swelling of the face, eyes, and hands. The white blood cell count was 8,800, with 82 per cent polymorphonuclear cells. An iodine patch test was positive. The iodoform pack was removed. The patient's symptoms cleared within twenty-four hours following administration of 5 grains of sodium chloride every four hours.

**CASE 4.**—Thrombocytopenic purpura following the application of iodine percutaneously.

A. G., a 55-year-old Italian male, developed pains in his right shoulder and left ankle. He applied a thick layer of tincture of iodine to both areas. Several hours later he developed purpuric spots all over his extremities, chest, and abdomen. There was marked swelling of both hands, as well as of the face, and especially over those areas where iodine had been painted. There was no palpable liver or spleen, and the temperature was normal. The platelet count was 80,000. The bleeding time was fifteen minutes, coagulation time was five minutes, and clot retraction was still absent the following day. The white blood cell count was 8,200, with 78 per cent polymorphonuclear cells. There was a positive tourniquet test. An iodine patch test read the following day showed marked redness. On the assumption that this was a case of thrombocytopenic purpura, based on an allergic reaction to iodine, this man was given 5 grains of sodium chloride every four hours. The purpuric spots steadily cleared up, the redness in his face disappeared, and the swelling of both hands slowly receded. One week later the platelet count was 140,000, the bleeding and coagulation times were within normal limits, and there was good clot retraction in two and one-half hours.

About two weeks after the initial episode, he came to the office feeling very well and asked to have a Wassermann test done. An iodine swab was accidentally used to sterilize the patient's antecubital fossa preparatory to venipuncture. This was immediately swabbed with alcohol. Eight hours later the patient had a chill, swelling of the skin of the antecubital fossa, and a reappearance of purpuric rash. The platelets were again depressed to 70,000, the bleeding time was prolonged to ten minutes, the coagulation time to five minutes, and there was absence of clot retraction. These findings again cleared up after 5 grain sodium chloride capsules every four hours for three days. Within ten days the platelets had returned to 200,000, and the bleeding and coagulation times became normal. The Wassermann reaction was negative.

**CASE 5.**—Cutaneous eruption following the application of tincture of iodine.

A. B., a 15-year-old girl, developed a fever of 102° F., redness and swelling of the hands and face following the application of tincture of iodine to a bruise on the skin. The bruise was surrounded by an erythematous, raised red area, resembling erysipeloid. Diagnosis was made after the patient gave a history of a previous similar experience three years

before. The iodine patch test was markedly positive. Urine determinations for iodine and iodides were negative. The symptoms cleared up promptly after several doses of sodium chloride in 5 grain capsules.

#### COMMENT

Considering the widespread use of iodine and iodides, the incidence of allergic reactions to these substances appears to be low. That they must be considered, however, is brought out by the many deaths reported in the literature. Hollander and Fetterman<sup>1</sup> reported the eleventh case of fatal allergic reaction in 1936. Since that time there have been at least seven more recorded instances. In our series we were fortunate in not having deaths, although some of the reactions were quite severe.

Three of our cases showed general reactions to cutaneously applied tincture of iodine. One developed a scarlatiniform eruption, another had a history of such an eruption many years before. The third patient developed a case of thrombocytopenic purpura, typical in all respects save enlargement of the spleen. The literature on hypersensitivity to the cutaneous application of iodine is covered in Seymour's article,<sup>2</sup> in which he adds a very instructive case. The patient was tuberculous, and iodine was applied prior to the institution of pneumothorax treatment. After several moderately severe reactions every time iodine was exhibited, a very severe reaction occurred which resulted in death. Before death the patient developed an exfoliative dermatitis.

The question of the use of iodoform packs following operation is a moot one. Cushny<sup>3</sup> stated that iodoform was almost useless as an antiseptic. He showed that microbes drawn from wounds under iodoform treatment were not retarded or weakened in their development. Indeed, he quoted work showing that the emigration of leucocytes from the blood vessels was hindered by the local action of iodoform. This would tend to retard healing. Iodoform slowly decomposes into iodine in the body, but the quantity so formed per unit time cannot be sufficient to sterilize the wound. It leads to a false sense of security, and when the question of sensitivity is taken into account, one wonders whether its use is justifiable.

The incidence of thrombocytopenic purpura following iodine allergy has not been so adequately discussed. Osler<sup>4</sup> described a case which commenced twenty-four hours after the taking of 10 drops of compound of tincture of iodine. Dennig<sup>5</sup> described three cases, all in elderly women with Basedow's disease. One followed the taking of potassium iodide in 6 drop doses daily for three weeks. Purpura developed. The platelets dropped to 20,000. Several weeks later the platelets increased and the patient finally recovered. Another of his cases is confused because of the coincident use of sedormid, together with tablets of an iodide compound. A third patient developed purpura following the consumption of sodium iodide for a short time. Both of these patients subsequently recovered.

There are several other cases with hemorrhagic symptoms in the literature, but unfortunately blood studies are lacking. Lobsenz<sup>6</sup> reported fatal iodine poisoning following the use of iodoform gauze drains. He attributed the severe

hemorrhagic symptoms that the patient developed to vitamin C deficiency, but this appeared to be an impression unsupported by laboratory tests. Rowell<sup>7</sup> reported a case showing hemorrhagic blebs on the extremities. The clotting time was between six and seven and one-half minutes. No figures were available on the platelet count.

All our cases showed local cutaneous sensitivity to tincture of iodine on patch test. This consisted of marked reddening and itching, perceptible in five to six hours. When observed at twenty-four hours, the erythematous area usually had extended slightly and was edematous. Since this reaction was invariable, irrespective of the method of intake of iodine or its salts in these patients, it was used as a method of detecting sensitivity, and called the iodine patch test. Jacobs<sup>8</sup> found essentially the same result in his case, although he used other iodine products and iodine ointment as well. However, in an unduly sensitive person, even one drop of tincture of iodine might conceivably cause a marked reaction. Hence it might be better to wipe off the iodine with some alcohol after a minute. Since our patients did not have marked reaction to one drop, we have not as yet used this precaution.

In our patients, the dose of iodine taken into the body was so small that qualitative tests for iodine and iodides in the urine were negative. Sodium chloride in 5 or 10 grain doses appeared to ameliorate the symptoms. This appears to be the usual method of treatment. One author<sup>9</sup> used sodium chloride intravenously. There are several reports of the use of epinephrine.<sup>10</sup> If the mechanism is indeed one of allergy, this latter treatment seems more logical. The rationale of the sodium chloride treatment is that the chlorine ion displaces the weaker iodine from the iodide. In very severe cases the use of adrenal cortex hormone, because of its action on the sodium chloride in the blood, would seem to be indicated along with the other means at our disposal.

The mechanism of iodine allergy is unknown. The Wolf-Eisner theory of the combination of iodine with a protein in the body to produce the antigen is not substantiated by Jacobs. This theory, although intriguing, still awaits proof.

The patient who has iodine allergy must be told of his condition. Failure to do so may place his life in jeopardy at a later date. The physician who plans to use iodine or one of the iodides in diagnosis or treatment should ask the patient whether he has any iodine sensitivity. The ideal procedure would be to patch test every prospective user of these compounds. However, this is not often possible because of the time element involved.

#### SUMMARY

1. Five cases of hypersensitivity to iodine and its salts are described.
2. A patch test for the detection of this sensitivity is described, and was present in all five patients.
3. Several types of cutaneous eruption followed the use of iodine or its salts in these patients, appearing in from eight to twelve hours. Scarlatiniform eruptions developed in two, and a purpuric rash in another.

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177 KINGSTON AVENUE

## A BACTERIOLOGIC CHECK ON RESIDUAL DENTAL INFECTIONS\*

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THAT the mere removal of infected teeth does not always suffice to eradicate dental infection has been taught by several authors. Martin H. Fischer's recent book, *Death and Dentistry*,<sup>1</sup> clearly re-emphasizes the problem. Removal of infected teeth alone "cures" the constitutionally afflicted in only some one-third of the cases, and "improves" the state of another third. There is abundant clinical and roentgenologic evidence that when teeth are extracted in the ordinary fashion, residual infection in the surrounding alveolar tissues may be left in place. Root fragments and bone rarefactions are conceded to be a source of systemic infection by most oral pathologists, but we would here emphasize the still larger importance of the infection remaining in the alveolar structures when such grosser evidences of pathology are nonexistent.

We have added to clinical and x-ray examination a bacteriologic study of cultures made from the alveolar structures. X-ray photographs of the full mouth were taken of patients presenting constitutional signs of chronic infection, after other possible sources for systemic invasion had been ruled out. These films showed an irregularity and furriness of the alveolar processes assumed by us to be caused by residual infection. The matter is illustrated by Figs. 1 and 2. In all the patients observed the teeth had been extracted at least five years previous to this study.

In a first series of edentulous patients who showed signs of residual infection by x-ray, as described above, a general anesthetic (pentothal sodium) was administered intravenously. When it had taken effect, the jaws were held open and the alveolar areas to be examined were scrubbed with soap and water.

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This was followed by a topical application of tincture of iodine, then alcohol, and finally, tincture of merthiolate. Swab cultures made from the surfaces thus prepared were negative in 8 of 10 patients studied, the remaining two patients showing organisms in no way related to the organisms cultivated from the

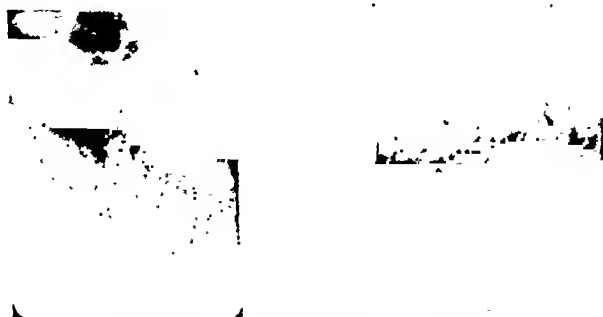


Fig. 1.—Note furriness and irregularity of the alveolar processes of a patient from whom all cultures made by puncture or grinding yielded a streptococcus.

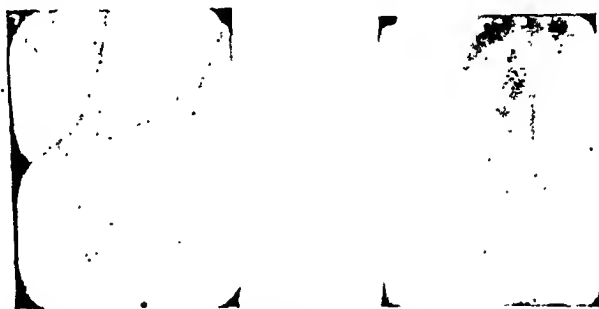


Fig. 2.—Note smooth contour of mandibular surfaces in comparison with Fig. 1. Stab and incision cultures from this patient proved negative.

underlying structures. After this sterilization of the surface, 1 c.c. of sterile saline solution was injected into the alveolar processes, allowed to remain there a few moments, and then withdrawn without removal of the needle in the interim. We could thus recover approximately 0.25 c.c. This was squirted into sterile tryptose broth. Twenty-four hours later subcultures were made. In the 10 patients showing no more than the alveolar furriness described above, organisms were recovered from all. In a control series of 6 patients presenting smooth alveolar ridges, cultures taken in a like manner always proved negative.

In a second series of 16 patients incision was made, and the alveolar bone was exposed after proper precedent cleansing. This time cultures were made of the bone grindings. Growth of organisms was obtained in every instance. The grinding was done with a sterile burr. Check of the organisms recovered at times from the buccal surfaces against those recovered from the alveolar processes showed them to be wholly nonrelated.

The foregoing indicates that the removal of teeth is not synonymous with the eradication of a locus of infection, that mere irregularity and furriness in contours of after-remaining alveolar processes as revealed by the x-ray are evidence of the continued existence of such a locus.

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# THE SIGNIFICANCE OF ELECTROCARDIOGRAPHIC CHANGES IN MALIGNANT HYPERTENSION\*

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## INTRODUCTION

THE changes that occur in the electrocardiogram of patients who have essential hypertension have been studied extensively. The exact nature of these changes is not fully understood, and there is much disagreement as to their significance.

In an attempt to clarify the controversy, a study of the fatal cases of malignant hypertension recognized at the Medical College of Virginia over a seven-year period was undertaken. Care was exercised in the selection of cases in order to eliminate any extraneous factors, such as valvular defects or coronary thrombosis, which might be present. Thirty-seven cases were available for study, comprising 54 electrocardiograms and 16 autopsy examinations.

The malignant phase of hypertension was chosen for several reasons. The course of the disease is very rapid, telescoping into a few months or years the changes that occur in essential hypertension over many years. The short time interval reduces the possibility of accidental occurrences which might alter the electrocardiogram. The disease usually terminates before senile degenerative changes have begun to appear. In practically all of these cases the patient died of uremia. Heart failure was not clinically present to complicate the electrocardiographic picture. It may be stated categorically that the changes seen in these electrocardiograms and observed at the autopsy table arose because of the presence of malignant hypertension.

## THEORETICAL CONSIDERATIONS

Many authors have noted changes in the electrocardiogram which occur when there is marked left ventricular preponderance. Master<sup>1</sup> states that there is a definite relationship between the appearance of these changes and the size of the heart, and implies that the size of the heart is the sole factor involved. Barnes and Whitten<sup>2</sup> suggest that the size of the heart may be responsible for some of the changes, but not all, and that certain changes in the T wave are indicative of "left ventricular strain" or myocardial damage. In this study an attempt has been made to correlate post-mortem findings with the electrocardiographic changes.

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Willius<sup>1</sup> was among the first to provide indication that in hypertensive heart disease there is a change in the T wave. He found that in cases exhibiting T-wave negativity in Lead I, or in Leads I and II, the predominant disease was hypertension. This was not true of other combinations of T-wave changes. Barnes and Whitten<sup>2</sup> found that where lesions increased the work of the left ventricle, inversions of the T wave were often present in Lead I, or in Leads I and II; similarly, right ventricular preponderance produced T-wave inversion in Leads II and III, although the correlation was not as close as in left ventricular preponderance. They found that in nearly two-thirds of the patients with simultaneous inversions of the T wave in all leads there was no evidence of ventricular preponderance. From this they concluded that the factors producing ventricular preponderance were not the same as those producing T-wave inversions. They hypothesized further that the changes were evidence of ventricular strain, and were not dependent on the size of the heart.

Master<sup>4</sup> holds a different opinion; he contends that the changes occurring in arterial hypertension are due solely to the increase in the size of the heart over a rather long period of time. His study consists largely of correlations between the electrocardiograms of patients with essential hypertension and the size of the heart as determined by the cardio-thoracic ratio in the teleroentgenogram. He states<sup>4</sup> that, as enlargement of the left ventricle begins, left axis deviation develops in the electrocardiogram. With further increase in the size of the left ventricle, T-wave inversion in the first lead occurs, followed by inversion of the T wave in the second lead. Roesler and co-workers<sup>5</sup> noted a steady increase in the percentage of cases with electrocardiographic final deflection changes as the radiologic examination showed an increase in the size of the heart, but they found that an absence of final deflection changes and of left axis deviation was compatible with a marked degree of cardiac enlargement. They state that some factor other than cardiac enlargement is necessary to produce these changes.

Odel<sup>6</sup> found structural changes in varying degrees of severity in the arterioles of the myocardium in a series of 35 cases of malignant hypertension. The presence of diffuse fibrosis in the myocardium in these cases added further evidence to the supposition that the electrocardiographic changes were due to myocardial damage. The electrocardiograms of these patients revealed depression of the S-T segment in Leads I or II in 59 per cent, and abnormal T waves in those leads in 47 per cent. Gouley<sup>7</sup> states that myocardial pathology is a common occurrence in uremia with severe hypertension, while Wood and White<sup>8</sup> attribute associated electrocardiographic changes to the toxic effect of uremia on the myocardium.

#### PRESENT STUDY

The present study may be divided into several parts. They consist of a consideration of the predominant trends in the electrocardiograms of 37 patients who died of malignant hypertension; the correlation of T-wave changes to myocardial damage as proved at autopsy; the relationship of cardiac weight to cardio-thoracic ratio; the relationship of myocardial damage to cardio-thoracic ratio; and the prognostic significance of the changes observed.

## I. PREDOMINANT CHANGES IN THE ELECTROCARDIOGRAM

A detailed analysis of all important features of the electrocardiograms in the cases studied is shown in Table I. The total number of electrocardiograms showing the particular changes are noted under each heading. From this basic chart Table II is derived. It was felt that all abnormalities of the T wave should be considered, and we believe that the results justify this procedure. A total of 75.9 per cent of the electrocardiograms showed abnormalities of the T wave in Lead I and/or Lead II. Left axis deviation was present in 59.3 per cent, while depression of the S-T segment in Lead I and/or Lead II was found in 50 per cent.

TABLE I

FUNDAMENTAL DATA: ANALYSIS OF 54 ELECTROCARDIOGRAMS IN 37 PATIENTS

| CHANGES  | LEADS |    |     |           |            |          |                |
|--|-------|----|-----|-----------|------------|----------|----------------|
|  | I     | II | III | I AND III | II AND III | I AND II | I, II, AND III |
| Notched P  | 0     | 3  | 1   | 1         | 4          | 0        | 1              |
| Diphasic P   | 0     | 1  | 0   | 0         | 0          | 0        | 0              |
| Negative P   | 2     | 0  | 3   | 0         | 0          | 0        | 0              |
| High voltage P                                       | 0     | 2  | 2   | 0         | 2          | 0        | 0              |
| High voltage R                                       | 8     | 3  | 0   | 0         | 0          | 2        | 2              |
| High voltage QRS                                     | 1     | 2  | 0   | 0         | 0          | 0        | 5              |
| Deep S   | 0     | 1  | 25  | 1         | 11         | 0        | 0              |
| Q wave   | 8     | 0  | 0   | 0         | 0          | 0        | 0              |
| Depressed S-T  | 9     | 7  | 0   | 0         | 3          | 11       | 0              |
| Starred QRS  | 2     | 3  | 10  | 5         | 2          | 0        | 3              |
| Elevated S-T   | 0     | 0  | 9   | 0         | 0          | 0        | 1              |
| Prolonged S-T  | 0     | 0  | 0   | 0         | 0          | 0        | 1              |
| Convex S-T   | 5     | 1  | 1   | 0         | 1          | 0        | 0              |
| Concave S-T  | 0     | 0  | 3   | 0         | 0          | 1        | 0              |
| Depressed S-T, upright T                             | 3     | 4  | 0   | 0         | 0          | 1        | 0              |
| Depressed S-T, negative T                            | 7     | 7  | 0   | 0         | 1          | 4        | 0              |
| Elevated S-T, upright T                              | 0     | 1  | 3   | 0         | 0          | 0        | 0              |
| Elevated S-T, negative T                             | 1     | 0  | 3   | 0         | 0          | 0        | 0              |
| Low T  | 7     | 2  | 3   | 3         | 2          | 1        | 0              |
| Flat T   | 2     | 3  | 0   | 1         | 1          | 0        | 0              |
| Diphasic T   | 8     | 8  | 3   | 1         | 3          | 2        | 4              |
| Negative T   | 5     | 1  | 2   | 1         | 1          | 2        | 1              |
| Total T changes                                      | 22    | 14 | 8   | 6         | 7          | 5        | 5              |
| Left axis deviation was present in 32, absent in 22. |       |    |     |           |            |          |                |

TABLE II

RELATIVE INCIDENCE OF PREDOMINANT CHANGES IN THE ELECTROCARDIOGRAMS OF 37 PATIENTS WITH MALIGNANT HYPERTENSION (54 EKG)

|  | PER CENT |
|--|----------|
| Changes (low, flat, diphasic, or negative) in T <sub>1</sub> , T <sub>2</sub> , or T <sub>1</sub> and T <sub>2</sub>   | 75.9     |
| Deep S <sub>1</sub> , or S <sub>2</sub> and S <sub>3</sub>   | 69.3     |
| Left axis deviation  | 59.3     |
| Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , or S-T <sub>1</sub> and S-T <sub>2</sub>   | 50.0     |
| Deep S <sub>2</sub>  | 46.3     |
| Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , or S-T <sub>1</sub> and S-T <sub>2</sub> and negative T <sub>1</sub> , T <sub>2</sub> , or T <sub>1</sub> and T <sub>2</sub> | 33.3     |
| High voltage R <sub>1</sub> , R <sub>2</sub> , or R <sub>1</sub> and R <sub>2</sub>  | 24.1     |
| Elevated S-T <sub>1</sub>  | 16.7     |
| Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , or S-T <sub>1</sub> and S-T <sub>2</sub> with upright T complexes in those leads   | 14.8     |
| Q wave, Lead I   | 14.8     |

These results show that in malignant hypertension the variations in the T wave and the S-T segment must be considered a concomitant of the disease. When S-T depression is associated with abnormalities of the T waves, it means there is a more severe effect of the disease upon the heart than the T-wave changes alone would indicate.

## II. ELECTROCARDIOGRAPHIC CHANGES AND MYOCARDIAL DAMAGE

In view of the controversy as to the presence or absence of myocardial damage in cases showing T-wave changes in the electrocardiogram when hypertension is present, we have analyzed the cases in our records which were autopsied. The high incidence of myocardial damage in cases showing T-wave changes in the electrocardiogram, and the absence of changes in cases where no damage was demonstrable, show that the changes indicate myocardial damage. This is demonstrated in Table III. A positive correlation is present in 68.75 per cent of cases.

TABLE III

CORRELATION OF T-WAVE CHANGES IN LEADS I AND/OR II WITH PRESENCE OR ABSENCE OF MYOCARDIAL DAMAGE ON POST MORTEM (16 CASES)

|                              | CASES | PER CENT |
|------------------------------|-------|----------|
| Damage with EKG changes      | 7     | 43.75    |
| Damage without EKG changes   | 2     | 12.50    |
| EKG changes without damage   | 3     | 18.75    |
| No damage and no EKG changes | 4     | 25.00    |

A positive correlation is present in 68.75 per cent of the cases, and a negative correlation in 31.25 per cent. While these figures are derived from too small a series to prove the point beyond question, they confirm the impression that the T-wave changes in malignant hypertension are due to myocardial damage, and not to enlargement of the heart.

## III. CARDIAC WEIGHT, MYOCARDIAL DAMAGE, AND CARDIO-THORACIC RATIO

Fourteen patients were available for analysis of the cardiac weight. Nine of these had a determination of the cardio-thoracic ratio before death. The mean cardiac weight was 586 Gm., and the mean cardio-thoracic ratio was 55 per cent. The deviations from the mean were considerable, the smallest heart weighing 400 Gm. and the largest 820 Gm., while the cardio-thoracic ratio ranged between 45 and 66 per cent. The interesting part of the analysis is the lack of correlation of the size of the heart to the cardio-thoracic ratio. There are many reasons for this, as shown in the footnote to Table IV.

We have analyzed our data in 20 cases in which the cardio-thoracic ratio was known. T-wave changes indicative of myocardial damage were present in 15 of these cases; the cardio-thoracic ratios in these cases ranged between 45 and 66 per cent, covering the entire range of our series. On the other hand, electrocardiograms showing no T-wave changes in five cases were also found to cover the entire range of cardio-thoracic ratios. The results are tabulated in Table V.

These findings suggest that there is no definite correlation of cardiac size with cardio-thoracic ratio, nor of electrocardiographic changes with cardio-thoracic ratio. The cardio-thoracic ratio is a totally unreliable guide in interpreting the changes in the electrocardiogram. It cannot be assumed that a high cardio-thoracic ratio means that the electrocardiographic final deflection changes are without significance.

TABLE IV  
CORRELATION OF CARDIAC WEIGHT TO CARDIO-THORACIC RATIO

| CASE NO. | CARDIAC WEIGHT<br>(GM.) | C-T RATIO<br>(%) | WEIGHT DEVIATION<br>FROM MEAN | RATIO DEVIATION<br>FROM MEAN |
|----------|-------------------------|------------------|-------------------------------|------------------------------|
| S4234F   | 400                     | 52               | -186                          | - 3                          |
| S13460F  | 470                     | 45               | -116                          | -10                          |
| S52132M  | 500                     | 45               | - 86                          | -10                          |
| S38192M  | 500                     | 56               | - 86                          | 1                            |
| S42537F  | 530                     | --               | - 56                          | ---                          |
| S40224M  | 550                     | 66               | - 36                          | 11                           |
| S13584M  | 580                     | 60               | - 6                           | 5                            |
| S32122F  | 590                     | 58               | 4                             | 3                            |
| M51122F  | 600                     | 55               | 14                            | 0                            |
| M27562M  | 610                     | --               | 24                            | ---                          |
| S4019M   | 650                     | --               | 64                            | ---                          |
| S32128M  | 660                     | 54               | 74                            | - 1                          |
| M45348M  | 750                     | --               | 164                           | ---                          |
| S45189M  | 820                     | 59               | 234                           | 4                            |

The mean cardiac weight is 586 Gm. The mean cardio-thoracic ratio, as determined by teleroentgenogram is 55 per cent. Statistical analysis of this very small series shows that, although there is a positive correlation, it is not greater than that which could occur by chance (coefficient of correlation is 0.435, standard error of coefficient of correlation is 0.354). Factors modifying the C-T ratio, such as degree of dilatation of the ventricles, the rotation of the heart in the body, and the anteroposterior diameter of the chest, render this an unreliable method of determining the true size of the heart.

TABLE V  
RELATIONSHIP BETWEEN T-WAVE CHANGES INDICATIVE OF MYOCARDIAL DAMAGE AND THE  
CARDIO-THORACIC RATIO IN THE SAME PATIENTS

| MYOCARDIAL DAMAGE | CARDIO-THORACIC RATIO<br>PER CENT |
|-------------------|-----------------------------------|
| No                | 45                                |
| Yes               | 45                                |
| Yes               | 51                                |
| Yes               | 51                                |
| No                | 52                                |
| Yes               | 52                                |
| Yes               | 53                                |
| Yes               | 54                                |
| Yes               | 55                                |
| Yes               | 55                                |
| Yes               | 55                                |
| No                | 56                                |
| No                | 58                                |
| Yes               | 59                                |
| Yes               | 60                                |
| Yes               | 60                                |
| Yes               | 61                                |
| Yes               | 64                                |
| Yes               | 66                                |
| No                | 66                                |

As shown above, no correlation exists between the cardio-thoracic ratio and T-wave changes indicative of myocardial damage.

#### IV. THE PROGNOSTIC SIGNIFICANCE OF THE ELECTROCARDIOGRAM OF MALIGNANT HYPERTENSION

It is recognized that the prognosis of malignant hypertension is serious, death usually occurring a few months to a few years after the disease has been discovered. It is still impossible to predict for the individual patient whether or not he will have the greater life expectancy of one or two years.

In an attempt to provide a more exact prognosis, we studied the electrocardiograms of each patient who had more than one electrocardiogram taken before he died, with special regard for the changes in the S-T segment and

TABLE VI

CHANGES IN S-T SEGMENTS AND T WAVES IN REPEATED ELECTROCARDIOGRAMS ON 11 PATIENTS DYING FROM MALIGNANT HYPERTENSION

| CASE NO. | DATE  | TIME INTERVAL              | S-T AND T CHANGES  | DATE DIED | DAYS AFTER LAST EKG |
|----------|---|----------------------------|--|-----------|---------------------|
| M51122F  | 4/18/40<br>4/25/40                          | 7 days                     | None<br>Elevated S-T <sub>1</sub> , S-T <sub>2</sub> , S-T <sub>3</sub> ,* flat T <sub>1</sub> , low T <sub>2</sub>  | 5/ 4/40   | 10                  |
| S79386M  | 3/21/40<br>4/18/40                          | 28 days                    | Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , diphasic T <sub>3</sub> , T <sub>1</sub><br>Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , concave S-T <sub>3</sub> , low T <sub>1</sub> , T <sub>2</sub> , diphasic T <sub>3</sub>   | 5/31/40   | 43                  |
| S82984F  | 8/27/40<br>9/ 2/40                          | 5 days                     | Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , low T <sub>1</sub> , T <sub>2</sub><br>Low T <sub>1</sub> , negative T <sub>2</sub>  | 9/14/40   | 12                  |
| S52132M  | 5/13/38<br>9/ 2/38<br>11/22/38              | 4 months<br>2½ months      | Diphasic T <sub>1</sub><br>Elevated S-T <sub>2</sub><br>Low T <sub>1</sub> , diphasic T <sub>2</sub> , T <sub>3</sub>  | 11/22/38  | 0                   |
| M45348M  | 10/20/37<br>10/22/37                        | 2 days                     | Depressed S-T <sub>2</sub> , low T <sub>1</sub> , T <sub>2</sub><br>Diphasic T <sub>1</sub>  | 11/ 4/37  | 13                  |
| S32122F  | 1/ 2/36<br>1/12/36                          | 10 days                    | Depressed S-T <sub>1</sub> , negative T <sub>1</sub> , diphasic T <sub>2</sub><br>Low T <sub>1</sub> , diphasic T <sub>2</sub>   | 1/18/36   | 6                   |
| S25555F  | 2/13/35<br>2/19/35                          | 7 days                     | Depressed S-T <sub>1</sub> , diphasic T <sub>1</sub> , T <sub>2</sub> , T <sub>3</sub><br>Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , low T <sub>1</sub>   | 2/24/35   | 5                   |
| S26791M  | 4/18/35<br>4/29/35<br>5/15/35               | 11 days<br>16 days         | Depressed S-T <sub>1</sub> , negative T <sub>1</sub> , T <sub>2</sub> , T <sub>3</sub><br>Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , low T <sub>1</sub> , diphasic T <sub>2</sub> , T <sub>3</sub><br>Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , diphasic T <sub>2</sub> , T <sub>3</sub> , low T <sub>1</sub>   | 5/21/35   | 6                   |
| S24735F  | 12/26/34†<br>12/31/34<br>1/ 3/35<br>1/ 8/35 | 5 days<br>3 days<br>5 days | Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , concave S-T <sub>3</sub> , S-T <sub>2</sub> , convex S-T <sub>3</sub> , low T <sub>1</sub> , flat T <sub>2</sub> , T <sub>3</sub><br>Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , elevated S-T <sub>3</sub> , flat T <sub>1</sub> , T <sub>2</sub> , diphasic T <sub>3</sub><br>Convex S-T <sub>1</sub> , flat T <sub>1</sub> , T <sub>2</sub> , negative T <sub>3</sub><br>Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , elevated S-T <sub>3</sub> , negative T <sub>1</sub> , diphasic T <sub>2</sub> | 1/10/35   | 2                   |
| S19858M  | 4/25/34<br>5/ 3/34<br>5/11/34               | 8 days<br>8 days           | Diphasic T <sub>1</sub> , flat T <sub>2</sub><br>Depressed S-T <sub>1</sub> , S-T <sub>2</sub> , convex S-T <sub>3</sub> , diphasic T <sub>1</sub> , T <sub>2</sub> , T <sub>3</sub><br>Depressed S-T <sub>1</sub> , diphasic T <sub>1</sub> , T <sub>2</sub> , flat T <sub>3</sub>  | 5/15/34   | 4                   |
| S12460F  | 5/14/34<br>9/24/35                          | 16 months                  | Negative T <sub>2</sub><br>None  | 11/ 1/35  | 38                  |

\*Consistent with pericarditis.

†Digitalis effect is present in this electrocardiogram.

Eighty-one per cent of these patients showed changes in the S-T segment or T wave characteristic of malignant hypertension in at least one electrocardiogram. All of them died in uremia; nevertheless, the electrocardiographic changes have considerable prognostic significance. Malignant hypertension is a diffuse arteriolar disease. When the coronary arterioles have become involved to a degree sufficient to produce myocardial damage, renal failure is near.

T wave. Eleven cases were available, with 27 electrocardiograms. It was found that 81 per cent of these patients showed characteristic S-T or T changes in at least one electrocardiogram. This may be explained, since the single electrocardiogram in the other cases might not have been taken at the most opportune time to demonstrate the changes. As shown by Bellet and McMillan,<sup>9</sup> terminal uremic pericarditis often elevates the S-T segment in all leads. It is likely that in some of our cases it raised the depressed S-T segment to the isoelectric line. The study indicates that in a very large majority of cases, characteristic electrocardiographic changes can be demonstrated at some time during the patient's life.

There was no uniformity in time from the appearance of the characteristic electrocardiogram to the death of the patient. However, as shown in Table VI, the appearance of these changes meant that death was near. When the widespread vascular process had proceeded long enough to produce changes in the heart muscle sufficient to give the characteristic electrocardiographic picture, the kidneys were usually on the verge of complete failure. While the electrocardiogram cannot predict the exact life expectancy, its changes indicate a more serious prognosis for the immediate future than does the normal electrocardiogram with the same degree of hypertension.

TABLE VII

FREQUENCY OF DEATHS FROM MALIGNANT HYPERTENSION AT VARIOUS AGES, GROUPED AT FIVE-YEAR INTERVALS

| AGE IN YEARS | NUMBER DYING |
|--------------|--------------|
| 15-19        | 1            |
| 20-24        | 2            |
| 25-29        | 3            |
| 30-34        | 7            |
| 35-39        | 10           |
| 40-44        | 8            |
| 45-49        | 6            |

Table VII shows that the greatest mortality is after the third decade, with no deaths after 50 years of age. This indicates that the changes found in the electrocardiograms of these patients are due almost entirely to the malignant hypertension. The degenerative senile processes, which may alter the electrocardiographic picture in essential hypertension, have not yet made their appearance.

Finally, in evaluating the significance of the electrocardiographic changes in malignant hypertension, a tabulation was made of the age at which each patient died (Table VII). The greatest mortality was in the group 35 to 39 years old. The majority of deaths occurred after the third decade was reached, while none occurred over 50 years of age. This indicates that the electrocardiogram is a reliable guide in following the progress of malignant hypertension, and has some prognostic value. Senile degenerative changes, which may modify its usefulness, have not appeared. It is evident that the characteristic changes observed in this series are due solely to the effect of the malignant hypertension on the heart.

#### CONCLUSIONS

1. Low, flat, diphasic, or negative T waves and depressed S-T segments are characteristically found in Lead I, Lead II, or Leads I and II of the electrocardiogram in malignant hypertension.



2. These changes indicate the presence of myocardial damage, and are only coincidentally related to the cardiac size.

3. The cardio-thoracic ratio is not a reliable guide in interpreting the electrocardiographic picture, nor has it any prognostic value.

4. The presence of typical changes in the electrocardiogram indicates a serious prognosis for the immediate future.

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## TEMPERATURE STUDIES ON INTRAVENOUS FLUIDS\*

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THE purpose of this presentation is to show the temperature at which preheated or precooled fluid actually enters the vein of the patient, and not to dictate at what temperature such fluid shall be given.

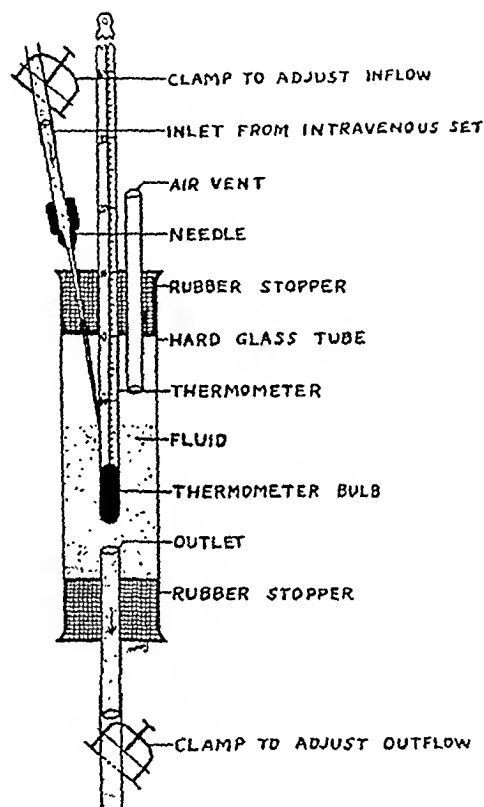


Fig. 1.—Details of thermometer assembly.

For the experimental work a standard intravenous set was used, and the fluid (normal saline) was allowed to fall one meter through regular gum rubber tubing (5 mm. diameter). A No. 20 needle was used. The needle was attached to the lower end of a Taylor Centigrade thermometer ( $-10$  to  $+150^{\circ}$  scale) by a rubber band, so that the fluid flowed directly out of the needle onto the thermometer bulb. The fluid was allowed to collect in a one liter flask, the thermometer bulb resting on the bottom of same. The flow was adjusted so that one hour was consumed in allowing one liter to run out. The temperature of the fluid was adjusted from  $+10$  to  $+90^{\circ}$  C. in  $10^{\circ}$  intervals. Three different arrangements were used originally, similar to the ones actually used in this hospital by various

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physicians. First, no extra external heat was applied after the solution was preheated. Second, two regular hot water bottles, containing 1,500 c.c. each of water at 55° C. (usual temperature of hot water in the taps in this hospital), were tied around the flask containing the fluid. Third, the tubing was "sandwiched" between two hot water hottles, 15 cm. from the needle. This last arrangement corresponds to the hot water bottles lying on the bed beside the patient's arm.

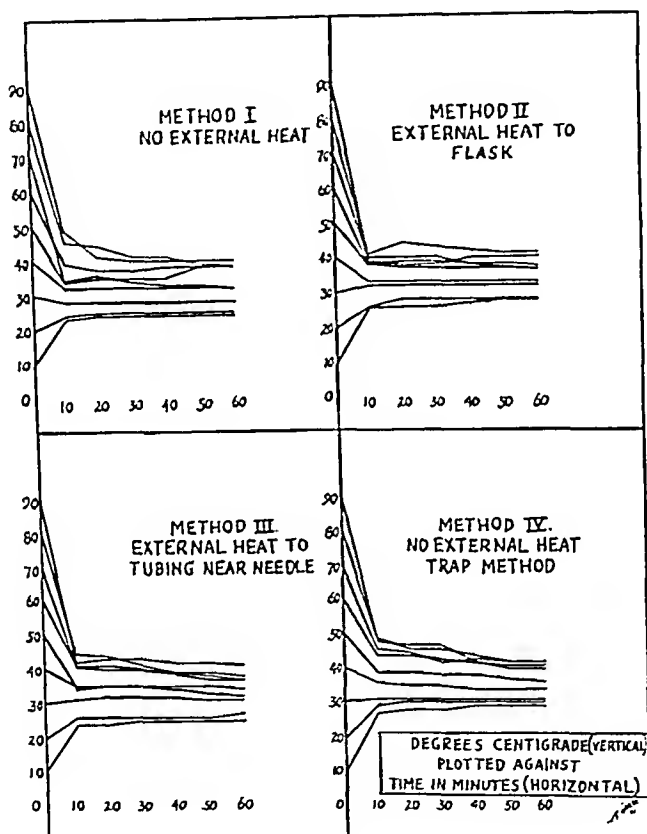


Fig. 2.

An attempt was made to read the temperature at the end of the first minute, but due to cooling by evaporation, and also due to the fact that not enough fluid had collected in the flask to cover the thermometer bulb, the readings were not considered accurate enough to include in the final report.

A fourth method was tried, in which the thermometer was inserted in a trap as shown in Fig. 1. The results are summarized in Fig. 2.

#### CONCLUSIONS

1. Preheated or precooled intravenous fluids tend to adjust themselves rapidly to a figure near room temperature, no matter what may be the original temperature of the original solution.

2. The customary methods of keeping intravenous fluids "warm" by the application of external heat in the form of hot water bottles are ineffectual.

# A NOTE ON THE INEFFECTIVENESS OF SULFONAMIDE COMPOUNDS IN THE TREATMENT OF POLIOMYELITIS OF MICE\*

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ALTHOUGH sulfanilamide and other sulfonamide compounds have been found ineffective in the treatment of experimental poliomyelitis of monkeys,<sup>1-5</sup> we have thought it advisable to determine whether or not they possess any therapeutic properties in poliomyelitis of mice.

Two hundred and thirty adult white mice (*Mus musculus*) were inoculated intracerebrally with 0.03 c.c. of a 5 per cent suspension in saline solution of the hind brains of mice removed after the development of flaccid paralysis following inoculation with the Lansing strain of poliomyelitis virus which has been adapted to these animals by Armstrong<sup>6</sup> from the eastern cotton rat (*Sigmodon hispidus hispidus*). While the virus has certain marked similarities to, as well as marked differences from, the spontaneous mouse virus first described by Theiler,<sup>7</sup> Lillie and Armstrong<sup>8</sup> have found that the pathologic changes produced by the virus in the central nervous system of mice and rats were closely similar to the reactions of poliomyelitis virus in man and monkeys.

As shown in Table I, 20, or 50 per cent, of 40 untreated controls developed paralysis and died within two to eight days after inoculation; 17, or 42.5 per cent developed paralysis and died in from ten to twenty-four days after inoculation, while only 3, or 7.5 per cent, escaped infection. All 37 of the 40 controls developing infection died within one to two days after the onset of flaccid paralysis of one or more of the legs or of the respiratory center, so that the disease in mice is so rapidly fatal after paralysis has developed that it is not well adapted for treatment purposes, although the incubation period following the intracerebral inoculation of virus is sufficiently prolonged in the majority of mice to permit the administration of repeated doses of compounds for the determination of therapeutic effects.

Sulfanilamide, sulfapyridine, and sulfathiazole, as well as two new compounds, sulfathiazoline (2-sulfanilyl 3-5 dihydrothiazole) and 2-sulfanilyl-aminopyrazine (No. 3032), synthesized by Raiziss and Clemence,<sup>9</sup> and kindly supplied by them, were administered orally in a dose of 0.005 Gm. per mouse twice daily (10:00 A.M. and 3:00 P.M.) for ten days in succession, or until paralysis and death occurred (2 to 20 doses). The first dose of each was given twenty-four hours after inoculation. As the mice averaged from 20 to 25 Gm. in weight, each dose was equivalent to about 0.2 Gm. per kilogram, or about 0.4 Gm. per kilogram per day.

\*From the Research Institute of Cutaneous Medicine, Philadelphia.  
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TABLE I  
SULFONAMIDE COMPOUNDS IN THE TREATMENT OF POLIOMYELITIS OF MICE\*

| COMPOUNDS†      | NO.<br>MICE | PARALYSIS AND DEATHS; DAYS AFTER INOCULATION |   |   |   |    |    |    |    |    |    |    |    |    | NO<br>PARALYSIS;<br>SURVIVALS |
|-----------------|-------------|--|---|---|---|----|----|----|----|----|----|----|----|----|-------------------------------|
|                 |             | 2  | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 |                               |
| Sulfanilamido   | 40          | 3  | 4 | 2 | 2 | 1  | 1  | 5  | 3  | 4  | 4  | 3  | 3  | 0  | 5 (12.5%)                     |
| Sulfapyridino   | 40          | 8  | 8 | 3 | 5 | 2  | 1  | 2  | 2  | 2  | 2  | 1  | 1  | 0  | 3 (7.5%)                      |
| Sulfathiazolo   | 40          | 2  | 6 | 2 | 2 | 4  | 3  | 1  | 5  | 3  | 3  | 4  | 1  | 0  | 4 (10.0%)                     |
| Sulfathiazolino | 40          | 4  | 8 | 2 | 0 | 6  | 4  | 0  | 6  | 6  | 0  | 2  | 0  | 0  | 2 (5.0%)                      |
| No. 3032‡       | 30          | 4  | 6 | 2 | 2 | 2  | 2  | 3  | 1  | 3  | 2  | 1  | 1  | 0  | 2 (5.0%)                      |
| Controls        | 40          | 3  | 8 | 5 | 4 | 2  | 2  | 2  | 2  | 4  | 1  | 2  | 2  | 0  | 3 (7.5%)                      |

\*Inoculated intracerebrally with 0.03 c.c. of a 5 per cent suspension of the Lansing strain adapted to mice (Armstrong).

†Orally in dose of 0.005 Gm. per mouse twice daily (10:00 A.M. and 3:00 P.M.), first dose twenty-four hours after inoculation.

‡2-sulfamthyl-amino-pyrazine.

As previously stated, 3, or 7.5 per cent, of 40 untreated controls survived. Of 40 mice treated with sulfanilamide, 5, or 12.5 per cent, survived; of 40 treated with sulfapyridine, 3, or 7.5 per cent survived; of 40 treated with sulfathiazole 4, or 10 per cent, survived; of 40 treated with sulfathiazoline, 2, or 5 per cent, survived, while of 30 treated with 2-sulfanilyl-aminopyrazine (No. 3032), 2, or 5 per cent, survived.

The administration of the compounds was continued after the development of paralysis, but with completely negative results, since all treated and untreated paralyzed animals died within one to two days after the onset of paralysis, permitting the administration of only one to three additional doses.

#### CONCLUSION

While the percentage of survivals among mice treated with sulfanilamide and sulfathiazole were slightly higher than that in the case of untreated controls, it is concluded that these compounds, as well as sulfapyridine, sulfathiazoline, and 2-sulfanilyl-amino-pyrazine, have proved ineffective in the treatment of mice inoculated intracerebrally with the Lansing strain of poliomyelitis virus (Armstrong). These results are in confirmation of the ineffectiveness of the sulfonamide compounds in the treatment of monkeys inoculated intracerebrally with the M.V. and other strains of the virus.

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# CLINICAL CHEMISTRY

## THE CHANGES IN THE BLOOD VOLUME PRODUCED BY DIABETIC ACIDOSIS\*

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ALTHOUGH it has been recognized that diabetic acidosis is associated with hemoconcentration,<sup>1</sup> very few observations have been made on the volume of circulating blood under these circumstances.<sup>2, 3</sup> During studies on the relation of dehydration to the plasma volume, observations were made on two patients who developed diabetic acidosis under controlled conditions.

### METHODS AND PROCEDURES

Blood volume determinations were made with the method of Gibson and Evans,<sup>4</sup> as modified by Gibson and Evelyn.<sup>5</sup> Venous pressure measurements were made by the direct method,<sup>6</sup> using 10 cm. above the level of the back as the point of zero pressure. Serum protein measurements were made by the falling-drop method described by Barbour and Hamilton.<sup>7</sup> The carbon dioxide combining power of the plasma was determined by the method of Van Slyke and Cullen.<sup>8</sup>

The patients observed were both males with diabetes who had previously been treated with diet and insulin for many years. Both were controlled on a 4,000 calorie experimental diet, consisting of 100 Gm. of protein, 265 Gm. of fat, 300 Gm. of carbohydrate, and insulin. After control values of the blood volume, venous pressure, serum protein concentration, plasma carbon dioxide combining power, and body weight were determined, insulin was discontinued. Daily urinalyses were done for sugar and ketone bodies.

### RESULTS

Patient 1 was observed without insulin for nine days. Although the entire 4,000 calorie diet was regularly consumed, there was a 5 kg. weight loss during this period. There was a loss of 1.5 kg. during the first twenty-four hours coinciding with the onset of severe glycosuria. Ketonuria appeared on the fourth day and gradually increased in severity. During the night of the eighth day nausea and vomiting developed, followed in the morning by drowsiness, Kussmaul respirations, anuria, low blood pressure, clammy extremities, and

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TABLE I

| PATIENT | DATE    | PERIOD          | WEIGHT (KG.) | VENOUS PRESSURE (MM. WATER) | TOTAL BLOOD VOLUME | PLASMA VOLUME | TOTAL BLOOD VOLUME/KG. BODY WT. | PLASMA VOLUME/KG. BODY WT. | HEMATOCRIT | SERUM PROTEIN GM./100 C.C. | CARBON DIOXIDE COMBINING POWER | KETONURIA |
|---------|---------|-----------------|--------------|-----------------------------|--------------------|---------------|---------------------------------|----------------------------|------------|----------------------------|--------------------------------|-----------|
| 1       | 2/18/41 | Control         | 62.3         | 100                         | 3,745              | 2,370         | 60.1                            | 38.1                       | 36.75      | 6.36                       | 67.3                           | Negative  |
|         | 2/18/41 | Without insulin |              |                             |                    |               |                                 |                            |            |                            |                                | Negative  |
|         | 2/19/41 | Without insulin | 60.8         |                             |                    |               |                                 |                            |            |                            | 64.2                           | Negative  |
|         | 2/20/41 | Without insulin | 59.5         |                             |                    |               |                                 |                            |            |                            | 62.6                           | Positive  |
|         | 2/21/41 | Without insulin | 60.5         |                             |                    |               |                                 |                            |            |                            |                                | Positive  |
|         | 2/25/41 | Without insulin | 58.6         |                             |                    |               |                                 |                            |            |                            |                                |           |
| 2       | 2/26/41 | Without insulin | 57.3         |                             | 2,885              | 1,500         | 52.0                            | 26.2                       | 48.00      | 7.72                       | 14.0                           | Positive  |
|         | 4/23/41 | Control         | 61.4         | 110                         | 3,560              | 2,135         | 58.0                            | 34.8                       | 40.00      | 6.18                       | 62.5                           | Negative  |
|         | 5/ 6/41 | Control         | 72.2         | 130                         | 6,100              | 3,660         | 84.4                            | 50.7                       | 40.00      | 6.71                       | 57.0                           | Negative  |
|         | 5/ 6/41 | Without insulin |              |                             |                    |               |                                 |                            |            |                            |                                |           |
|         | 5/10/41 | Without insulin | 71.8         | 136                         | 6,020              | 3,600         | 83.8                            | 50.1                       | 40.20      | 6.89                       | 60.0                           | Positive  |
|         | 5/15/41 | Without insulin | 69.4         | 134                         | 5,820              | 3,240         | 83.8                            | 46.7                       | 44.30      | 7.47                       | 47.5                           | Positive  |
|         | 5/20/41 | Without insulin | 67.1         | 100                         | 5,200              | 2,860         | 78.4                            | 42.6                       | 45.00      | 8.05                       | 26.0                           | Positive  |
|         | 6/10/41 | Control         | 73.2         | 140                         | 6,360              | 3,560         | 86.9                            | 48.6                       | 44.80      |                            | 52.2                           | Negative  |



marked collapse of the veins. The plasma carbon dioxide combining power at this time had fallen to 14.0 volumes per 100 c.e. The arterial blood pressure, usually 130/80, was now 90/60. The veins were collapsed.

There was a severe reduction of 860 c.e. (23 per cent) in the total blood volume due entirely to a 36 per cent decrease in plasma volume. In spite of the loss of 5 kg. of weight, there was a marked decrease in the blood volume and plasma volume per kilogram of body weight from 60 c.e. to 52 c.e. (13.4 per cent) and from 38 c.e. to 26 c.e. (31.2 per cent), respectively. The hematocrit and serum protein concentrations were increased 30.6 per cent and 21.4 per cent, respectively. These changes are recorded in detail in Tables I and II. There was a prompt recovery within eight hours following the intravenous administration of 4,000 c.e. of 5 per cent glucose in normal saline, insulin, sodium bicarbonate, and forced fluids by mouth.

Patient 2, who was not as severe a diabetic as Patient 1, was observed without insulin for fifteen days. Although the high caloric intake was maintained, there was a 5.1 kg. loss in weight, most of which occurred after the plasma carbon dioxide combining power had decreased.

TABLE II  
PERCENTILE CHANGES

| PATIENT | % CHANGE<br>BODY<br>WEIGHT | % CHANGE<br>IN TOTAL<br>BLOOD<br>VOLUME | % CHANGE<br>IN PLASMA<br>VOLUME | % CHANGE<br>IN TOTAL<br>BLOOD<br>VOLUME/KG.<br>BODY WT. | % CHANGE<br>IN PLASMA<br>VOLUME/KG.<br>OF BODY WT. | % CHANGE<br>IN<br>HEMATO-<br>CRIT | % CHANGE<br>IN CON-<br>CENTRATION<br>OF SERUM<br>PROTEINS |
|---------|----------------------------|---|---------------------------------|---|--|-----------------------------------|---|
| 1       | -8.0                       | -23.0                                   | -36.2                           | -13.4   | -31.2  | +30.6                             | +21.4   |
| 2       | -5.7                       | -14.7                                   | -21.8                           | - 7.1   | -15.9  | +12.5                             | +20.0   |

By the fifteenth day there was an 800 c.e. (21 per cent) decrease in plasma volume and a marked reduction in total blood volume and plasma volume per kilogram body weight from 84 c.e. to 78 c.e. (7.1 per cent) and from 51 c.e. to 43 c.e. (15.9 per cent), respectively. The loss of blood volume was largely at the expense of the plasma volume; the hematocrit and serum protein concentrations were increased 12.5 per cent and 20 per cent, respectively. The venous pressure was reduced from 130 mm. to 100 mm. of water (23 per cent). These changes are recorded in detail in Tables I and II. The patient promptly recovered with the administration of insulin, fluids, and glucose by mouth.

#### DISCUSSION

The data presented here indicate that the blood volume is significantly decreased in diabetic acidosis, the reduction being entirely at the expense of the plasma volume. Both the total blood volume and the plasma volume per kilogram body weight were also reduced. There was an increase in the hematocrit and plasma protein concentration that closely paralleled the decrease in plasma volume. These results are in agreement with those of Chang, Harrop, and Schaub,<sup>2</sup> who used the carbon monoxide method to determine blood volume and who found a reduction in the blood volume per kilogram body weight and an average decrease in plasma volume of 19 per cent. Peters<sup>1</sup> reported details

on 13 patients who had an average decrease of 16 per cent in the concentrations of serum proteins with recovery from diabetic acidosis. The large decreases in plasma volume in man, as determined by the dye method and the carbon monoxide method, as well as the changes in hematocrit and serum protein, indicate that the plasma volume shares an important part in the dehydration associated with diabetic acidosis.

Sundermann and Dohan,<sup>3</sup> using vital red dye for the measurement of blood volume, in a study of ketosis in 6 pancreatectomized dogs, found an average decrease of 10 per cent in total serum volume and an actual increase of 29 per cent in the serum volume per kilogram body weight. None of these dogs, however, developed a marked acidosis, as measured by the carbon dioxide content of plasma. It is possible that under these circumstances the acidosis was not sufficiently severe or prolonged to cause a greater depletion of the extracellular fluid. Atchley, Loeb, and others,<sup>9</sup> in a study of the electrolyte changes in two patients with diabetes, found that with the withdrawal of insulin there was an increase in the excretion of both potassium and sodium which would indicate a loss of both intracellular and extracellular fluid. They have pointed out that the initial electrolyte and water loss was dependent upon the sudden appearance of marked glycosuria.

The demonstration of the marked fall in blood volume in man associated with the decrease in venous pressure may well explain the cause for the peripheral circulatory collapse often seen in severe diabetic acidosis. It has been observed<sup>10</sup> that the diminution in plasma volume produced by mercurial diuretics or ammonium chloride acidosis is associated with a nearly proportionate decrease in venous pressure in normal persons. It has been established that changes in venous pressure in dogs and man will produce significant alterations in the cardiac output.<sup>11-15</sup> Thus in diabetic acidosis the low blood volume and its accompanying fall in venous pressure may produce a decrease in the cardiac output. The low arterial pressure, anuria, clammy extremities, and the clinical picture of peripheral circulatory collapse seen in diabetic acidosis may, therefore, be the result of the decrease in the volume of circulating blood.

The marked reduction in blood volume in diabetic acidosis, particularly when associated with peripheral circulatory collapse, should indicate immediate replacement of the blood volume as an important step in the management of this type of patient. Although there may be an increase in the concentration of serum proteins at the time of the acidosis, the parenteral administration of normal saline or glucose may not necessarily be expected to increase the plasma volume immediately,<sup>1</sup> since the fluid may be lost to dehydrated tissues. Blood transfusion, or better still serum transfusion, should be especially helpful in the replacement of plasma volume and the relief of the circulatory collapse.

#### CONCLUSIONS

1. Diabetic acidosis observed in two patients was associated with a marked reduction of blood volume due to the loss of plasma volume and a fall in blood volume per kilogram body weight.

2. Associated with the fall in blood volume there was also a significant fall in venous pressure.

3. It is emphasized that the circulatory collapse sometimes found in diabetic acidosis is in part at least the result of the accompanying decrease in blood volume and venous pressure.

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## THE VALUE OF BLOOD CHLORIDE AND SODIUM DETERMINATIONS IN THE DIAGNOSIS OF DEHYDRATION\*

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**I**N A typical case the syndrome of dehydration is not difficult to recognize. The patient shows dryness of the mouth and tongue; shocklike weakness and apathy; diminished skin turgor; sunken, softened eyeballs; lowered blood pressure, associated frequently with a rapid pulse rate and other signs of circulatory distress, and marked diminution in urinary output, which occasionally approaches anuria. Frequently, however, the clinical picture is not so straightforward as thus presented. It may be obscured by other symptoms associated with the disease producing the dehydration, or it may even be produced by the therapeutic measures undertaken to control the disease. Not infrequently, because the scanty urine may contain some albumin and a number of casts and even red blood cells, and may be associated with nitrogenous retention, the condition is diagnosed as acute nephritis. This diagnosis usually causes a paralysis of all efforts at controlling the dehydration.

Since severe clinical dehydration is always associated with a loss of salt, and since this loss is reflected in the concentration of chloride and sodium in the blood,<sup>1</sup> it should be possible to recognize the condition by the appropriate blood chemical determinations. It is the purpose of this paper to present evidence that determinations of whole blood chloride are usually misleading in the information they impart concerning the state of hydration, because the whole blood chloride concentration varies with the degree of the anemia. It will be shown also that, though serum chloride values are not affected by the red blood cell concentration and though they are a more satisfactory gauge of dehydration than whole blood chloride values, they are also at times unreliable; for the serum chloride may be normal or even higher than normal in many cases of excessive water loss. On the other hand, evidence will be offered to show that a lowering of serum sodium concentration always accompanies severe dehydration and that its determination, now feasible in all clinical laboratories, should be used in conjunction with serum chloride analysis in the diagnosis of dehydration.

### METHODS

To determine the influence of red blood cell concentration upon the whole blood and serum chloride levels, a study of the distribution of chloride between

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cells and serum (or plasma) was made in 80 persons. These persons were either healthy medical students or technicians or were ambulatory dispensary patients with varying degrees of anemia. No cases of pernicious anemia or other types of hyperchromatic anemia were chosen. No patient had any clinical or laboratory evidence of disturbance of water or electrolyte balance.

For the analysis, blood was drawn from an arm vein with a minimum of stasis into dry syringes. The portion for whole blood was immediately transferred to a tube containing potassium oxalate. For the determination of red blood cell volume percentage, Van Allen<sup>2</sup> hematocrit tubes were filled to the mark and diluted with 1.3 per cent sodium oxalate. The tubes were sealed with wide rubber bands and then centrifuged at 2,500 r.p.m. for at least twenty minutes, until the column of red blood cells no longer diminished in height. Blood for serum analysis was transferred under oil to centrifuge tubes, stoppered and allowed to clot, and then centrifuged for ten minutes. The serum was separated and recentrifuged to remove the last trace of red blood cells. Both whole blood and serum chloride determinations were made by the microphotoelectric iodate method of Sendroy,<sup>3</sup> as modified by one of us (W. S. H.).<sup>4</sup> All analyses were made in duplicate and averaged. Hematocrit estimations were made in triplicate. The red blood cell concentration was estimated from the equation

$$R = \frac{100 W - (100 - H) S}{H} \quad (1)$$

where  $R$  and  $S$  are the red blood cell and serum chloride concentrations, respectively;  $W$ , the whole blood concentration; and  $H$ , the volume percentage of red blood cells or the hematocrit reading.

For the determinations in the later clinical studies in this paper mentioned in Table II, the following methods were employed: sodium, Hoffman and Osgood;<sup>5</sup> carbon dioxide-combining power, Van Slyke;<sup>6</sup> protein and nonprotein nitrogen, Hoffman and Osgood;<sup>7</sup> hemoglobin, Sheard and Sanford.<sup>8</sup> In all the photoelectric methods the Cenco-Sheard-Sanford Photometer was used. The average normal concentration of serum sodium was found to be 143 meq. per liter, the range being 138 to 148 meq. per liter.

#### WHOLE BLOOD CHLORIDE

For the sake of brevity, the results of the analyses are expressed in frequency charts in Fig. 1 and as scatter curves in Fig. 2. Serum chloride concentration (Fig. 1a) varied from 100 to 110 meq. per liter, the great majority of values falling between 102 and 107 meq. per liter. The average serum chloride concentration was 104.4 meq. per liter, with a standard deviation of  $\pm 2.0$  meq. per liter ( $610 \pm 12$  mg. NaCl per 100 c.c.). The solid circles in Fig. 1 represent cases of anemia, that is, patients with hematocrit readings less than 40 per cent. It can be seen that there is no difference between the distribution of the serum chloride values of anemic persons and those with normal red blood cell volume. All the values fit into a compact probability curve. Similarly, the red blood cell chloride values are independent of the hematocrit reading (Fig. 1b). Their range is relatively wider than that of the serum

chloride values, and the probability curve arrangement is not so definite. However, this fault can be explained by the greater error of the calculation of the red blood cell chloride. An error of 1 meq. in either whole blood or serum chloride produces an error of 2 to 5 meq. in the red blood cell chloride concentration, depending upon whether the hematocrit reading is high or low. Nevertheless, the distribution of the values for anemia cases is the same as that for normal cases. The range is from 47 to 57 meq. per liter, with a mean value of 52.3, and a standard deviation of  $\pm 2.5$ .

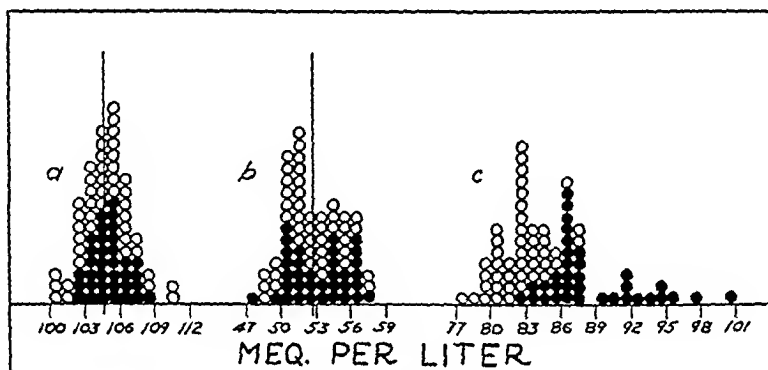


Fig. 1.—Frequency distribution of chloride concentrations in *a*, serum *b*, red blood cells and *c*, whole blood in 80 cases. Solid circles represent cases of anemia, that is, hematocrit readings of less than 40 per cent.

On the other hand, the values for whole blood chloride (Fig. 1c) are much more widely distributed than those for serum or red blood cells. The range is from 77 to 100 meq. per liter. The average value is 84.6 meq. per liter, and the standard deviation is  $\pm 4.6$  meq. per liter. However, the values fit so poorly into any probability curve that the mean value and the standard deviation have very little significance. The anemia cases are distributed all to the right side of the chart, indicating that the whole blood chloride is dependent upon the degree of anemia. If there had been more severe anemia cases, there would undoubtedly have been more weighting to the right, and an increase in the standard deviation.

If the red blood cell and serum chloride concentrations are independent of the hematocrit reading, then the whole blood chloride *must* vary with the hematocrit reading. This can be seen from equation (1), which may be re-written as

$$W = S - \frac{S - R}{100} H \quad (2)$$

where *S* and *R* (the serum and red blood cell chloride concentrations, respectively) are regarded as constants, and *W* and *H* (the whole blood chloride and the hematocrit) are variables. The lower the value of *H*, the higher the value of *W*. When the hematocrit becomes zero, the whole blood chloride equals the serum chloride, a conclusion which is obvious without reference to the equation. Choosing *S* as 104.4 meq. per liter, and *R* as 52.3 meq. per liter, equation (2) becomes the line AB in Fig. 2a, where *W* is the ordinate and *H* is the abscissa. This is the curve of expected values for whole blood chloride,

based on the assumption of the constancy of the serum and red blood cell chloride concentrations. The curve is an oblique line passing through the base ( $H = 0$ ) at  $W = 104.4$ . The observed values for whole blood chloride, plotted against the hematocrit with the same coordinates as the curve AB, form the scatter curve which follows closely the theoretical curve. The deviations from the points on the theoretical curve are no wider than those in the scatter for serum chloride plotted along with the theoretical equation,  $S = 104.4$  (line AC, Fig. 2b).

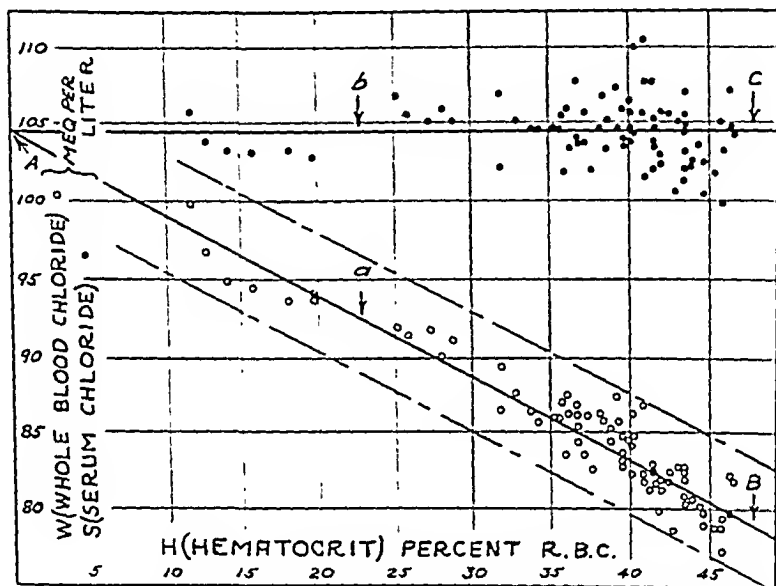


Fig. 2.—a, Scatter curve showing the relationship between whole blood chloride concentrations and the hematocrit readings in 80 cases. The line AB represents the theoretically expected curve. b, Scatter curve showing the relationship between serum chloride concentrations and the hematocrit readings in the same 80 cases. The line AC represents the theoretically expected curve.

Thus these data offer statistical confirmation of the proposition first stated by Norgaard and Gram<sup>9</sup> that the serum and cell chloride concentrations are independent of the degree of anemia, but that the whole blood chloride concentrations must necessarily vary with the red blood cell volume percentage. Failure to recognize this fact has made whole blood chloride determinations worthless in most clinical laboratories. It would probably be better that the determination be abandoned altogether for clinical purposes. But where it is carried out, the expected normal range must be determined from a chart such as Fig. 2a. For this purpose, Table I has been constructed from the data in Fig. 2a and from the hemoglobin concentrations determined in the 80 cases. The range of probable values for any given hematocrit was determined by drawing two lines parallel to the theoretical curve AB, so as to include all the observed values. Table I should be of aid in recognizing low blood chlorides in those cases where such a finding confirms the diagnosis of dehydration. The following two cases illustrate the failure to interpret properly whole blood chloride determinations.

TABLE I

VARIATIONS OF WHOLE BLOOD CHLORIDE WITH THE HEMOGLOBIN CONCENTRATION AND RED BLOOD CELL VOLUME IN BLOOD OF PATIENTS WITH NO ELECTROLYTE DISTURBANCE

| HEMOGLOBIN<br>GM./100 C.C. | HEMATOCRIT<br>% R.B.C. | WHOLE BLOOD CHLORIDE |            |                      |            |
|----------------------------|------------------------|----------------------|------------|----------------------|------------|
|                            |                        | RANGE OF NORMAL      |            | EXPECTED MEAN        |            |
|                            |                        | MG.<br>NaCl/100 C.C. | MEQ./LITER | MG.<br>NaCl/100 C.C. | MEQ./LITER |
| 13-16                      | 40-47                  | 456-509              | 78-87      | 479                  | 82         |
| 11-13                      | 35-40                  | 474-526              | 81-90      | 497                  | 85         |
| 9-11                       | 30-35                  | 491-544              | 84-93      | 514                  | 88         |
| 8-9                        | 25-30                  | 503-555              | 86-95      | 532                  | 91         |
| 7-8                        | 20-25                  | 520-573              | 89-98      | 544                  | 93         |
| 3-7                        | 10-20                  | 532-602              | 91-103     | 567                  | 97         |
| 0 (Serum)                  | 0                      | 585-643              | 100-110    | 608                  | 104        |

CASE 1.—A girl of 21 was brought to the hospital suffering from fever, jaundice, repeated vomiting, and oliguria, following an attempted abortion. The patient did abort, but her condition grew worse. The urine was scanty and showed some albumin, red blood cells, and several casts, and there was some nitrogenous retention. A diagnosis of acute nephritis was considered. The hemoglobin was 10 Gm. per 100 c.c., and the whole blood chloride was 450 mg. of NaCl per 100 c.c. This value was regarded as within normal limits, since the range of whole blood chloride is usually given as 450 to 500 mg. of NaCl per 100 c.c. When it was pointed out that the expected whole blood chloride for such a degree of anemia was 514 mg. of NaCl per 100 c.c. and that the probable lower limit for normal was 491 mg. of NaCl per 100 c.c. a serum chloride analysis was made. The serum chloride concentration was 538 mg. of NaCl per 100 c.c. or 92 meq. per liter, a value 11 per cent below the average normal. The patient was given 4 liters of physiologic saline intravenously within twenty-four hours. She began to urinate and made a complete recovery.

CASE 2.—A man aged 65 years, five days after a prostatectomy, developed secondary hemorrhage. Following this accident, the quantity of urine diminished rapidly, and the blood chemical analysis showed increasing nitrogenous retention. The patient had been on a low salt diet previous to the operation and had little salt following the operation. The condition was diagnosed as uremia following renal failure. The hemoglobin concentration was 8.9 Gm. per 100 c.c., and the whole blood chloride concentration was 465 mg. of NaCl per 100 c.c., which was thought to be well within normal limits. But with this degree of anemia, the whole blood chloride should have been within the limits of 503 to 555 mg. of NaCl per 100 c.c. When this fact was made clear, intravenous saline therapy was instituted, and improvement soon followed. With adequate urinary output the blood nonprotein nitrogen concentration soon decreased to normal, and the patient eventually recovered.

#### SERUM CHLORIDE VERSUS SERUM SODIUM

The foregoing data unequivocally indicate the diagnostic advantages of serum chloride determinations over those of whole blood chloride. In many uncomplicated cases of dehydration, the serum chloride is sufficiently lower than normal to confirm the clinical diagnosis. However, this is not always true. The data assembled in Table II from our own cases indicate that serum chloride may be low, normal, or even high in proved cases of dehydration. The 15 cases in Table II were chosen to illustrate the findings in dehydration associated with a variety of disease processes. In all these cases the clinical symptoms of dehydration were pronounced, and clinical improvement followed immediately upon the intravenous injection of sufficient physiologic saline solution (usually along with Hartmann's solution) to restore the electrolyte balance to normal. The quantity of fluid required for an adult usually was about 4,000 c.c. in the first twenty-four hours.



TABLE 11  
BLOOD CHEMICAL FINDINGS IN 15 CASES OF DEHYDRATION

| CASE | DISEASE   | HEMOGLOBIN<br>GM./100 C.C. | HEMATOCRIT<br>% R.B.C. | SERUM<br>GLUCORIDE<br>MEQ./LITER | WHOLE<br>BLOOD<br>CHLORIDE<br>MEQ./LITER | SERUM<br>SODIUM<br>MEQ./LITER | CO <sub>2</sub> COM-<br>BINING<br>POWER<br>C.C./100 C.C. | SERUM<br>PROTEIN<br>GM./100 C.C. | S.P.S.<br>MG./100 C.C. | REMARKS   |
|------|---|----------------------------|------------------------|----------------------------------|--|-------------------------------|--|----------------------------------|------------------------|---|
| 3    | Chronic glomerulo-<br>nephritis<br>a. Before<br>treatment<br>b. After NaCl<br>therapy | 10.0<br>8.5                | 27.5<br>24.2           | 97.4<br>108.6                    | 82.9<br>92.0                             | 130<br>141.5                  | 47<br>55   | 5.90<br>5.50                     | 80<br>78               | Marked vomiting   |
| 4    | Acute glomerulo-<br>nephritis   | 12.9                       | 38                     | 90.2                             | 65.2                                     | 132.5                         | 57   | 5.66                             | 140                    | Marked oliguria and<br>azotemia                                     |
| 5    | Chronic glomerulo-<br>nephritis   | 9.2                        | 27.0                   | 123.2                            | 106                                      | 135                           | 27   | 4.06                             | 108                    | Marked acidosis due<br>to prolonged<br>NH <sub>4</sub> Cl ingestion |
| 6    | Acute glomerulo-<br>nephritis   | 7.4                        |                        | 87.6                             |  | 116                           | 46   | 5.28                             | 148                    | Anuria following<br>relapsing and<br>polyuria, vomiting             |
| 7    | Chronic glomerulo-<br>nephritis   | 6.6                        | 21.0                   | 107                              | 94.7                                     | 134                           | 34   | 6.75                             | 240                    | Hemorrhage, vomit-<br>ing, uremia                                   |
| 8    | Malignant nephro-<br>sclerosis  | 4.8                        | 14.5                   | 103.5                            | 95.0                                     | 134                           | 23   | 5.31                             |                        | Fever, vomiting,<br>diarrhea  |
| 9    | Acute hepatitis   |                            |                        | 96.5                             |  | 125                           | 45   | 9.34                             |                        | Dysentery   |
| 10   | Acute gastro-<br>enteritis  |                            |                        | 111                              |  | 127                           | 38   | 9.12                             |                        |   |
| 11   | Diabetic acidosis   |                            |                        | 101                              |  | 128                           | 23   | 7.48                             |                        | Polyuria  |
| 12   | Pyloric obstruction   |                            |                        | 91                               |  | 136                           | 84   | 8.10                             | 40                     | Vomiting, oliguria  |
| 13   | Gastrocolic fistula   |                            | 40.0                   | 85                               |  | 133                           | 88   | 6.78                             |                        | Diarrhea  |
| 14   | Addison's disease   | 12.0                       | 38.5                   | 100                              |  | 128                           | 52   | 6.60                             | 62                     | Polyuria, vomiting  |
| 15   | Addison's disease   | 11.8                       | 37.8                   | 110                              |  | 134                           | 48   | 6.28                             | 56                     | Polyuria, vomiting  |
| 16   | Diabetic acidosis   | 13.5                       | 41.0                   | 96                               | 82.0                                     | 128                           | 28   | 6.50                             | 44                     | Polyuria, vomiting  |
| 17   | Morenial<br>nephrosis   | 12.8                       | 38                     | 85                               | 68.2                                     | 117                           | 44   | 8.40                             | 86                     | Vomiting, diarrhea  |

In 8 of the 15 cases the serum chloride values, as well as the serum sodium values, were lower than normal, but in the remaining cases the chloride values were normal, at the upper limit of normal, or even higher than normal. When vomiting is a prominent symptom in the disease producing dehydration, chloride is usually depleted at the same rate as sodium. In those cases where partial restitution of electrolyte balance is attempted either unwittingly or therapeutically by ingestion of sodium chloride, chloride may rise to normal or above normal while the sodium concentration still remains low. This is particularly likely to occur in Bright's disease and in Addison's disease, when the kidneys, because of either organic or functional derangement, cannot readily excrete the excess chloride. Case 5 illustrates the development of dehydration and acidosis during an uncontrolled intake of ammonium chloride. Here, dehydration was present in spite of extremely high serum chloride concentration, yet the serum sodium value properly reflected the clinical state.

In all 15 cases the serum sodium concentration was lower than the minimal normal value of 138 meq. per liter. The deviation from normal was not very great, if one uses as a criterion the changes that can occur in urea or glucose concentrations. But even a 5 per cent drop below the average normal value indicates marked dehydration, for in the early stages of dehydration the sodium concentration still remains within normal limits. Even in the alkalosis of pyloric obstruction and gastroduodenal fistula (Cases 12 and 13) there was a sodium deficit. It was not as great as the chloride deficit, but it determined the degree of dehydration in these cases. In the first hours of pyloric vomiting the chloride may drop rapidly below normal, but not until the sodium loss, in the vomitus and in the urine, produces a serum sodium concentration lower than normal, is severe dehydration present.

When, as in Case 6, the serum sodium is as low as 116 meq. per liter, it is scarcely compatible with life. Along with the usual symptoms of dehydration, there is marked oliguria and azotemia. The symptoms and signs may be difficult to distinguish from true uremia. The patient, in this case, had had labor induced because of eclampsia. At the time of the blood chemical analysis she had scarcely urinated at all for several days. She was bloated, had pallid facies, was weak and apathetic, and was obviously in serious condition. The blood nonprotein nitrogen concentration was 108 mg. per 100 c.c., and the blood pressure was 170 mm. systolic and 100 mm. diastolic. In spite of the risk of recurrence of convulsions saline injections were recommended. The patient was given 3,000 c.c. of physiologic saline solution intravenously during the first twenty-four hours, and 2,000 c.c. the following day. The blood pressure rose slightly, but no convulsions occurred. The urinary output was 1,200 c.c. on the day following onset of treatment and rose to 3,000 c.c. daily. The nonprotein nitrogen dropped to normal, and the patient recovered completely in several weeks.

The data in Case 3 illustrate the changes in the blood chemistry after adequate water and salt therapy. The serum sodium, chloride, and carbon dioxide-combining power rose to normal value. At the same time the increase in plasma volume produced a dilution of hemoglobin, red blood cells, serum protein, and nonprotein nitrogen. Thus, though it was true that the period

of dehydration was associated with hemoconcentration, this could not have been ascertained at the time by analysis for hemoglobin, red blood cell volume, or serum protein, for all these values were well below normal. Yet the serum sodium and chloride indicated the true condition. (It should be noted that the whole blood chloride was within so-called normal limits.) Only in 4 of the 15 cases was the serum protein above the normal limits, even though hemoconcentration was undoubtedly present in all these cases.

The determination of serum sodium, it has been seen, may be of lifesaving importance. It should not necessarily replace the determination of serum chloride. The two analyses, along with that of the carbon dioxide-combining power, should be performed in all cases of suspected water and acid-base disturbance. The three determinations together give adequate information in severe cases of dehydration, not only in regard to the volume and mineral content of the extracellular fluids, but also with respect to the degree and type of acid-base balance disturbance. If, for example, the sodium and chloride are equally lowered and yet the carbon dioxide-combining power is low, then the acidosis is due to the retention of acid ions other than chloride. In nephritis these ions are almost always phosphate and sulfate, but in other diseases they are usually organic acids—acetoacetic and hydroxybutyric acids in diabetes and starvation; lactic acid in cardiac failure and in convulsions.

Moderate or incipient dehydration is not associated with changes in serum chloride or sodium. There is at present no laboratory aid for the detection of such early dehydration. However, if the dehydration is not severe enough to have produced electrolyte changes, it is seldom a factor in the differential diagnosis. Moreover, it is usually not difficult to recognize the condition by clinical observation. As Simeone<sup>10</sup> has recently pointed out: "Long before ionic changes are demonstrable in the blood, the withdrawal of interstitial fluid causes a diminution in the blood flow through the salivary glands, the secretions from which are thereupon impoverished. Thus the best index of early dehydration is the old clinical observation of dryness of the buccal and oral mucous membranes. Dysphagia is usually present. . . . The value of blood chemical determinations is not to be belittled, however; they at least demonstrate the degree of dehydration, and the nature of the shift in electrolyte pattern serves as a guide for replacement therapy."

#### SUMMARY

A study of the distribution of chloride between red blood cells and serum was made in 80 persons who had varying red blood cell volume percentages but who had no evidence of dehydration or electrolyte imbalance. The serum chloride ranged between 100 and 110 meq. per liter, with a mean value of 104.4 and a standard deviation of  $\pm 2.5$ . The red blood cell chloride concentration was also independent of the anemia. The whole blood chloride, however, varied directly with the red blood cell volume in a predictable manner. From the scatter curve of variations of the whole blood chloride with the hematocrit reading, a table was prepared which indicated the expected whole blood chloride value for any particular hemoglobin concentration or hematocrit reading. It was shown that failure to interpret whole blood chloride concentrations

according to such a table has prevented clinicians from recognizing dehydration, particularly when such dehydration was the cause of oliguria and nitrogenous retention.

In a study of 15 cases of severe dehydration in a variety of disease processes, serum sodium was found always lower than the minimal normal of 138 meq. per liter. In these cases, serum chloride, though at times correspondingly low, was occasionally within normal limits or even higher than normal.

The conclusion was reached that a proper evaluation of the water and mineral content of the body in dehydration, both from the point of view of diagnosis and therapy, can be achieved by analysis of the serum for sodium, chloride, and carbon dioxide-combining power.

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# THE EXPERIMENTAL PRODUCTION OF HYPOALBUMINEMIA BY FASTING\*

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ALTHOUGH the causal relationship of a protein-deficient diet and nutritional edema has been known since World War I, hypoproteinemia was not recognized as a crucial phenomenon, largely because there were few determinations of plasma protein in dietary deficiency until the extensive clinical observations of Peters and his co-workers,<sup>1</sup> beginning about 1926. A summary of most of their findings and a review of the literature was published by them in 1933.<sup>2</sup> Prominent among the contributions of Peters was, first, the fact that the fall in serum protein in malnourished patients was confined to the albumin fraction, the globulin remaining relatively unaffected; second, that the fall in serum protein might be masked by dehydration, the lowered value not becoming evident until the dehydration was corrected; third, various infections often produced a rise in serum globulin, thus creating a normal total serum protein, even though there was a depletion in the albumin fraction.

Experimental hypoproteinemia was induced with a protein-deficient diet by Frisch, Mendel, and Peters,<sup>3</sup> who were able to lower the serum protein in white rats to 3.0 Gm. per 100 c.c. Much more extensive observations on the dog were made by Weech and his co-workers,<sup>4</sup> who used a low protein diet, consisting of carrots, sucrose, lard, rice, cod-liver oil, and a salt mixture. Confirming the clinical observations of Peters, they noted that the fall in serum protein was confined to the serum albumin, the globulin fraction remaining unchanged. A second, and very important observation, was the fact that the fall in serum albumin began at once and continued progressively during the first month or more the animal was on the diet.

Though the influence of a complete fast on the concentration of serum protein has received considerable attention, the findings are much less conclusive. As early as 1882 Burekhardt<sup>5</sup> found that the concentration of total protein fell slightly when dogs were fasted for four days. Hanson,<sup>6</sup> in 1918, reviewed the earlier literature and concluded that "a wide disparity in the results obtained by different workers is the rule rather than the exception." Hanson himself studied six rabbits during alternate periods of fasting and feeding, the longest period of fasting being six days. Though no very marked differences were noted, there was a tendency for the albumin concentration to be slightly lower after the longer periods of fasting. Bloomfield<sup>7</sup> and Torbert<sup>8</sup> studied rats which were fasted, killed at intervals, and the serum protein determined. Though the total protein concentration dropped initially,

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the findings in general were not very conclusive. However, Torbert noted that the initial drop was in the albumin fraction; he found, furthermore, that the blood volume decreased during the experiment so that the total circulating protein diminished, even though the concentration remained relatively unchanged. The same finding was reported in a carefully controlled experiment by Holman, Mahoney, and Whipple<sup>9</sup> in a dog that was fasted for twenty-four days. These workers concluded that "fasting usually does not modify the total protein concentration in the plasma, and the albumin-globulin ratio may not be changed, but fasting does cause conspicuous shrinkage of the blood plasma volume and, therefore, the total amount of circulating protein may decrease to 70, 60, or even 50 per cent of normal."

During a complete fast there is, of course, an increased breakdown of protein in order to supply calories, thus leading to a release of large amounts of nitrogen, much of which may remain as amino acids. The idea has been expressed that this extra nitrogen might spare serum protein depletion or even lead to its regeneration. Indeed Weech and his collaborators,<sup>10</sup> and Melnick and his collaborators,<sup>11</sup> each found that during a period of starvation, there was an increase in the concentration of serum protein in a dog with chronic hypoproteinemia.

#### EXPERIMENTAL PROCEDURES

Two series of experiments were carried out in 15 dogs. In Experiment I 5 dogs were given by gavage for three weeks a solution containing Karo syrup, Ringer's solution, and vitamin B complex,\* and were allowed water *ad libitum*; the energy value was 500 calories per day, due entirely to carbohydrate, and nitrogen was 0.07 Gm. per day. In Experiment II 10 dogs were fasted completely; water was allowed *ad libitum*, but 3 did receive in addition Ringer's solution by gavage. Samples of heparinized blood were removed at the beginning of the experiment and each week thereafter by puncturing the jugular vein and removing a sample with as little stasis as possible. The red cell volume was determined after centrifugalization, and the supernatant plasma used for the determination of albumin and globulin, separation being carried out by the method of Campbell and Hanna.<sup>12</sup> Determinations of the nitrogen were carried out by a micro-Kjeldahl, as described by Sobel, Yuska, and Cohen.<sup>13</sup>

#### EXPERIMENTAL FINDINGS AND COMMENT

The present findings show that hypoalbuminemia occurs on a protein- and fat-deficient diet regardless of whether or not there is any caloric intake. Thus the serum albumin concentration declined both in the 5 dogs given a solution containing carbohydrate, Ringer's solution, and vitamin B (Table I), as well as in the 10 animals fasted completely (Table II). Moreover the changes were progressive and started from the very beginning of the experiment.

The average fall in the albumin was twice as great in the dogs receiving calories as in those completely fasted (29 per cent as compared with 14 per cent). Inferences cannot be drawn from this striking difference, which was due in part at least to hemoconcentration occurring in the fasted dogs, as

\*Labco was obtained from The Borden Company, Bainbridge, N. Y.

TABLE I

## HYPOALBUMINEMIA DURING A NONPROTEIN DIET

(Intake: Karo syrup, Water, Electrolyte, and Vitamin B)

A = Plasma albumin, grams per 100 c.c. H = Hematocrit as per cent.

G = Plasma globulin, grams per 100 c.c.

| DOG        | INITIAL |      |      | ONE WEEK |      |      | TWO WEEKS |   |   | THREE WEEKS |      |      |
|------------|---------|------|------|----------|------|------|-----------|---|---|-------------|------|------|
|            | A       | H    | G    | A        | H    | G    | A         | H | G | A           | H    | G    |
| Z-10       | 3.90    | 50   | 3.00 | 2.81     | 41.6 | 2.75 |           |   |   | 2.08        | 39.7 | 3.52 |
| Z-21       | 3.65    | 41   | 2.51 | 2.96     | 46.0 | 2.83 |           |   |   | 2.76        | 44.3 | 3.62 |
| Z-14       | 2.58    | 56   | 3.28 | 2.05     | 55.8 | 3.22 |           |   |   | 1.99        | 52.4 | 3.31 |
| T-67       | 3.03    | 57   | 2.80 | 2.41     | 53.7 | 3.36 |           |   |   | 2.22        | 52.0 | 3.10 |
| T-56       | 3.79    | 52   | 2.90 | 3.20     | 47.9 | 3.17 |           |   |   | 3.10        | 50.0 | 2.76 |
| Averages   |         |      |      |          |      |      |           |   |   |             |      |      |
| Albumin    | 3.39    |      |      | 2.62     |      |      |           |   |   | 2.13        |      |      |
| Hematocrit |         | 51.8 |      |          | 49.6 |      |           |   |   |             | 47.7 |      |
| Globulin   |         |      | 2.90 |          |      | 3.07 |           |   |   |             |      | 3.26 |

TABLE II

## HYPOALBUMINEMIA DURING COMPLETE STARVATION

(Intake confined to water *ad lib.* except Dogs 62, E6, and E2 who also received some Ringer's solution)

A = Plasma albumin in grams per 100 c.c. H = Hematocrit as per cent.

G = Plasma globulin, grams per 100 c.c.

| DOG        | INITIAL |      |      | ONE WEEK |      |      | TWO WEEKS |      |      | THREE WEEKS |      |      |
|------------|---------|------|------|----------|------|------|-----------|------|------|-------------|------|------|
|            | A       | H    | G    | A        | H    | G    | A         | H    | G    | A           | H    | G    |
| F-8        | 3.39    | 45.1 | 2.90 | 3.03     | 46.6 | 3.08 | 2.90      | 44.3 | 2.77 | 2.91        | 42.4 | 2.58 |
| 56F2       | 2.94    | 48.2 | 2.25 | 3.33     | 53.1 | 2.61 | 3.13      | 54.1 | 2.39 | 2.56        | 57.2 | 2.38 |
| F-23       | 2.61    | 42.9 | 4.87 | 2.53     | 42.1 | 5.58 | 2.28      | 44.4 | 5.34 | 2.34        | 48.8 | 5.37 |
| Z-10       | 2.88    | 47.1 | 3.12 | 3.28     | 51.4 | 3.30 | 2.93      | 57.9 | 3.12 | 2.66        | 51.7 | 3.05 |
| 52         | 3.14    | 53.5 | 3.52 | 2.89     | 48.2 | 3.37 | 2.68      | 49.5 | 3.20 | 2.44        | 46.2 | 4.12 |
| Z-8        | 3.92    | 51.4 | 3.02 | 3.64     | 54.2 | 2.73 | 3.28      | 56.0 | 2.57 | 3.31        | 57.0 | 2.73 |
| 59         | 3.26    | 51.1 | 2.17 | 3.22     | 51.0 | 2.66 | 2.84      | 49.2 | 2.52 | 2.76        | 43.2 | 2.52 |
| 62         | 3.73    | 52.6 | 3.32 | 3.03     | 49.6 | 2.92 | 2.96      | 49.7 | 2.71 | 3.18        | 61.0 | 3.63 |
| E6         | 2.65    | 48.8 | 2.75 | 2.46     | 48.3 | 2.99 | 2.28      | 47.9 | 3.04 | 2.23        | 44.0 | 3.01 |
| E2         | 3.64    | 46.7 | 2.78 | 3.36     | 44.8 | 2.70 | 3.10      | 49.8 | 2.65 | 3.07        | 50.4 | 2.56 |
| Averages   |         |      |      |          |      |      |           |      |      |             |      |      |
| Albumin    | 3.22    |      |      | 3.07     |      |      | 2.83      |      |      | 2.77        |      |      |
| Hematocrit |         | 48.7 |      |          | 48.9 |      |           | 50.2 |      |             | 50.1 |      |
| Globulin   |         |      | 3.07 |          |      | 3.19 |           |      | 3.03 |             |      | 3.19 |

indicated by comparing the behavior of the hematocrit values in the two groups. Thus, while the average red blood cell volume fell in Experiment I, there was a slight increase in Experiment II. Though there was considerable individual variations in the group of fasted animals, as can be seen by studying each of the experiments in Table II, the increase in the red blood cell volume was so pronounced in several instances that the fall in serum albumin not only was masked but even gave way to a rise. (See the italicized values in Table II.) This effect of dehydration on the concentration of serum albumin was first observed in human beings by Peters and co-workers,<sup>1</sup> as already mentioned.

One can only speculate on the influence exerted by caloric intake on the magnitude of the albumin loss in view of the striking influence of a complete fast on the red blood cell volume as just pointed out. Whether the protein-sparing action of glucose would affect the plasma as well as tissue protein cannot be answered from the present data; it would require experiments in which both the plasma volume and the nitrogen excretion were measured.

The independence of the globulin fraction of the plasma during the intake of the protein-deficient diet in these experiments confirms previous clinical observations of Peters,<sup>1</sup> experimental findings by Weech and his co-workers,<sup>4</sup> as well as other experiments from this laboratory.<sup>14, 15</sup> In other words, removal of protein and fat intake affects only the albumin fraction of the plasma, and not the globulin.

Worthy of mention is the fact that in the present experiments and in others,<sup>14</sup> as first shown by Weech and associates,<sup>4</sup> the fall in albumin begins at once, and is progressive, initially at least, as long as protein is withheld from the diet. If there is such a thing as "deposit protein," or "reserve protein," it certainly fails to manifest itself under the conditions of these experiments. It would seem, therefore, that hypoproteinemia due to deficiencies in dietary protein cannot be prevented by body protein, or some other source of endogenous protein, which suggests that the concept of reserve protein may, perhaps, not apply to hypoproteinemia produced by dietary means alone.

#### SUMMARY

1. A pronounced (29 per cent) fall in the concentration of the albumin fraction of the plasma was observed in 5 dogs on a protein- and fat-deficient diet for three weeks; a similar but much smaller (14 per cent) fall occurred in 10 other dogs that were fasted completely. The globulin fraction remained relatively unchanged.

2. The less pronounced hypoalbuminemia in the animals completely fasted was accompanied by an increased red blood cell volume and was, therefore, due in part at least to dehydration; in a few instances of pronounced hemoconcentration the decline was masked or even replaced by a rise.

3. The observed hypoalbuminemia began with the onset of the experiment and was progressive. This indicates the absence of any store of reserve protein for the manufacture of serum albumin during a dietary induced deficiency.

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# LABORATORY METHODS

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## GENERAL

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### SIMPLIFICATION OF FLUORESCENCE MICROSCOPY\*

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THE fluorescence method of microscopy for the demonstration of *Mycobacterium tuberculosis* has recently attracted some attention in this country, but a more general testing and appraisal of the procedure is likely to be delayed because of a belief that the apparatus required is elaborate and complicated, or else expensive and difficult to obtain. Furthermore, commercial equipment already under test in some laboratories has met a most unfavorable reaction on the part of the workers who use it because the illumination is so dim that the tubercle bacilli can be seen only in an almost dark room, and at the expense of great eyestrain during protracted examination of films. This is totally unnecessary, but unless a more satisfactory form of light is devised the whole method may fall into disrepute as impractical. The principle is so simple, however, and the application so easy that the method may well be given the investigation it deserves if the technique can be reduced to a minimum no more difficult than ordinary microscopic examination.

The principle is elementary. It has been found that organisms of the acid-fast group, when stained by any one of a series of yellowish dyes, retain the stain after decolorization with acid alcohol, while bacteria not acid-fast lose the stain. The dyes used for the staining, including auramine-O, berberine sulfate, and thioflavine, are those which fluoresce when illuminated by ultraviolet light just off the end of the visible spectrum, the so-called "glass ultraviolet," which passes through ordinary glass with only moderate reduction in intensity. If a film stained and decolorized as indicated is illuminated with a powerful light which has passed through a deep blue filter of appropriate transmission band, the organisms which have retained the stain fluoresce a bright orange yellow, while the rest of the field is dazzlingly blue. An orange-yellow glass disc on the diaphragm of the ocular, selected for color so that it passes the fluorescent light of the bacilli and is opaque to the blue light of the field, will allow the stained bacteria to flare out on a dark background. The appearance is much like that seen with dark-field illumination, though the principle is totally different, and the fact that the organisms are self-luminous from their fluorescence and in sharp contrast to their surroundings makes them appear much larger than usual, so that they are easily seen with a high dry objective, as of 4 or 8 mm. focal length.

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The descriptions of the fluorescence method in the German and American literature are likely to give the impression that a rather special form of lamp is needed to give the necessary ultraviolet output.<sup>1-6</sup> Since, however, the light passes through the ordinary substage condenser of the microscope, perhaps fully 2 cm. in thickness, as well as through a condensing lens on the light source and through the common glass slide, the ultraviolet requirements plainly cannot be very exacting. Likewise, the insistence that a polished front-surface aluminum mirror must be used in place of the usual microscope mirror, in the belief that a silver surface absorbs the ultraviolet, is unfounded. A powerful, concentrated source of light, like a carbon arc or a 500-watt projection bulb, fitted with a condensing lens of about 7 cm. focal length with the blue filter in front of the condenser will supply an illumination bright enough to permit examination of films in a room darkened only by drawing the window shades.

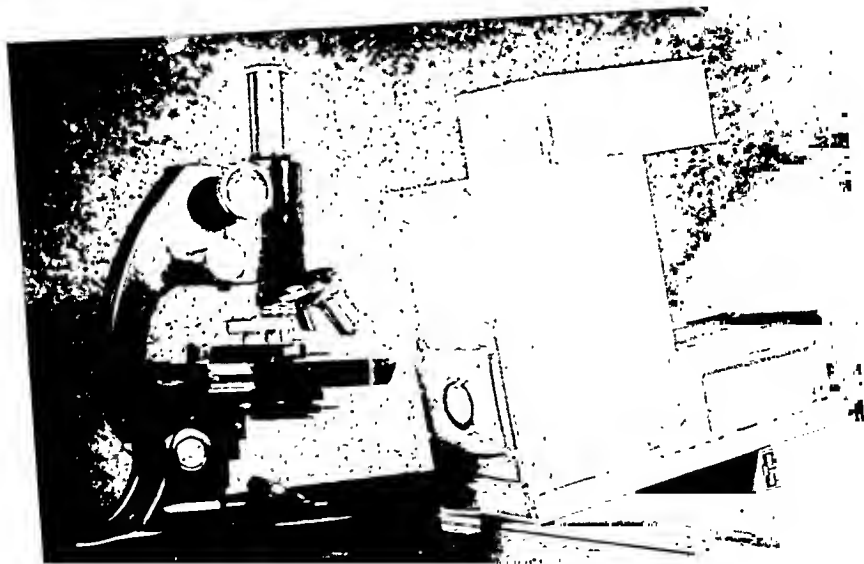


Fig. 1.

A satisfactory bulb for the purpose has been found in the 500-watt G. E. projection bulb operating on 110 to 115 volts. This is mounted in a ventilated sheet metal lamp box improvised in the laboratory. A condenser can be obtained from an old arc light, but a pair of ten-cent store reading lenses in tandem has proved equally useful. The proper relation of the lamp to the condensing lens is determined by trial and error, checking the effect of changes by the appearance of the field in the microscope. It has been found that the lamp should be set with the plane of the filament coils at an angle of about  $30^\circ$  to the optical axis so that the images of the coils will nearly overlap, as seen in the lighted field of the microscope. The housing should be large and provided with ample air inlet and outlet to dissipate the heat rapidly. It has also been found that the filter is better placed in front of the condensing lens rather than behind it. The beam of light should practically cover the mirror of the microscope, and its location is best seen when the mirror is half covered with a card during

adjustment. This arrangement calls for only an ordinary commercial bulb of relatively low cost, plus the application of a little ingenuity in making the housing, and dispenses with all special equipment, except the blue and yellow filters.\* A substitute for the blue filter can be made with an ammoniacal solution of cupric sulfate contained in a flat-sided bottle, and adjusted to the proper depth of color by watching the microscope field as the solution is diluted.

For the benefit of those who have not had the opportunity to consult the literature which outlines the fluorescence technique, the procedure is described in its simple detail.

Heat-fixed films are stained in the cold for three minutes with a solution of auramine-O (natrial aniline) made up as follows: auramine-O, 0.1 Gm., phenol absolute, 3 ml., and freshly distilled water, 97 ml. This solution should be kept in the dark, and made fresh every two weeks. It forms a slight precipitate without apparent loss of staining property. The film is rinsed in running water and decolorized for two minutes, and again for five minutes, with two applications of the solution: concentrated hydrochloric acid, 4 ml., sodium chloride, 4 Gm., 70 per cent alcohol, 1 liter. Decolorization may be continued much longer if desired, even for twenty-four hours. The slide is rinsed in running water, dried, and examined with the 4 mm. objective and a 10× ocular, or with an 8 mm. objective and a 20× ocular.

In films made from concentrates of sputum by sodium hydroxide digestion, practically no fluorescent material is seen, except the auramine-stained bacilli, so that a question often asked by persons seeing such a preparation for the first time will be, "Is this from a culture?" Direct films from sputum or spinal fluid, however, may contain many fluorescent inflammatory cells which hinder the examination. In such cases the suggestion of Hermann may be followed, to counterstain for a few seconds with 0.1 per cent potassium permanganate solution followed by Loeffler's methylene blue, to make the cells so dark a blue that they become invisible.<sup>2</sup> In this laboratory concentrate films are examined routinely, so that no difficulty has arisen, except in the case of the spinal fluid of tuberculous meningitis, in which instance the counterstain has worked perfectly. Since a properly stained preparation will show nothing fluorescing except the bacilli, if any are present, it may be difficult to focus on the plane of the slide with no reference object in view. To meet this difficulty the suggestion has been made that a dot or streak of the auramine stain be put on one end of the slide just before it is examined, to give a reference plane for focusing.

In adjusting the illumination, it has been found convenient to pick up the light from the plane mirror with the low-power objective and center it properly before the high dry lens is swung into use. If organisms are abundant, they can usually be seen with the low power directly, and if the low- and high-power lenses are parfocal, the final adjustment is much simplified. The low-power field appears dark red, greenish, or almost black with any fluorescent particles showing as bright dots of light. The condenser should be at its top position, as lowering it gives a red or light-colored background. The aluminized mirror, as stated, is not necessary, but the microscope mirror and condenser should be kept reasonably clean.

\*These may be obtained from the Spencer Lens Company.

The use of the immersion lens is unnecessary in routine examination of films, but it may be employed to check on the morphology of bacilli that appear atypical. Cedar oil, mineral oil, and sandalwood oil are not satisfactory as immersion media, since they all fluoresce and cast a haze over the field, but methyl salicylate (oil of wintergreen) is an excellent immersion oil with a high refractive index, and does not fluoresce. It can also be used as a mounting medium in the examination of tissue sections by the fluorescence method.

It has been noticed that an auramine-stained film will fade in the area exposed to the ultraviolet light if left in the field for about ten minutes. The fluorescence, of course, implies absorption of energy from the activating light, and apparently photochemical changes also take place. Likewise, stained films fade after the lapse of time, and the brightest preparations are those which have just been made, but restaining is satisfactory at any time. A Ziehl-Neelsen stain can be superimposed upon an auramine stain, but not vice versa.

Examination of fluorescence films stained with auramine-O is considerably easier than similar examination of Ziehl-Neelsen films, because of the wide field covered by the dry objective and because the organisms are either seen or not seen without confusion with their surroundings, provided the illumination is bright enough to cause brilliant fluorescence. This proviso is the reason the use of such a high-power lamp is recommended. Probably at least 95 per cent of the light from the bulb is stopped in the filter, and the near ultraviolet output of a filament bulb is not great in any case, so that a large initial energy input is needed if enough active light is to reach the film to cause bright fluorescence. Fortunately economy is not an important factor in the choice of a light source, and the high-power lamp, ready to operate the moment it is plugged in, answers all practical requirements. Once the light has been put together and the adjustments of mirror and condenser completed, the system can be used indefinitely if no changes of position are made.

A photograph of the improvised light housing is shown to indicate its general manner of construction. Other designs of housing or other types of bulb may prove even more useful. It is hoped that simplification of the technique may stimulate further test of this perhaps valuable method of investigation.

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# PRODUCTION OF EXPERIMENTAL HYPERTENSION AND THE INDIRECT DETERMINATION OF SYSTOLIC ARTERIAL PRESSURE IN RATS\*

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THE assay of antipressor extracts of kidney requires the use of large numbers of hypertensive animals. Dogs have been extensively employed, but their disadvantage is the high cost of care and the resultant limitation of the number of observations. Rats are cheaper, but many investigators have had difficulty both in producing hypertension and in measuring their arterial pressure. The purpose of this communication is to record the experience we have had in the preparation and study of over 1,000 hypertensive rats.

## PRODUCTION OF HYPERTENSION

Page (1939) described the use of silk and cellophane for eliciting hypertension by formation of a perinephric fibrocollagenous hull around the parenchyma of the kidneys. Thin China silk, organza silk, or even thin cotton batiste may be used. After anesthetizing the rat (males weighing 140 to 160 Gm. are most satisfactory) with seconal (sodium propyl-methyl-carbonyl allyl barbiturate, Lilly, 35 to 40 mg. per kilogram injected subcutaneously), the forelegs are tied down with the back of the animal against the operating board, while the hind legs are tied with the abdomen down, so twisting the animal in such a way as to give excellent exposure of the kidneys. The operator's hands and instruments are scrubbed, but aseptic technique is unnecessary. An incision is made parallel with the vertebral column, beginning in the costo-vertebral angle, and the kidney is delivered with the aid of chalazion forceps. The fat is stripped off, the silk is folded around the kidney and held in place by either a string around the pedicle or a purse-string ligature. The silk should be applied smoothly. The kidney is then replaced and the muscle and skin are closed with silk thread (No. A). Silk may be applied to both kidneys or only to one and its partner removed. Page, Patton, and Ogden (1941), and Williams, Harrison, and Grollman (1942) have modified this procedure by applying collodium instead of silk, but the latter investigators do not find it as satisfactory as silk.

We have also used the method employed by Drury (1938) for producing hypertension in rabbits, namely, constricting the main renal artery by a loop of silk thread. A wire approximately one-third the diameter of the normal artery

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(28 gauge wire is satisfactory for a 150 Gm. rat) is employed. A midline incision is made, the relative size of the renal artery is estimated with the help of a jeweler's loupe, and a wire is selected. The silk loop is placed around the wire and renal artery proximal to the suprarenal branch, and the wire is withdrawn. Since the left renal artery is the more accessible, this side is used when only one vessel is to be constricted. If the right artery is employed, the loop is placed proximal to the point at which the vessel runs dorsal to the inferior vena cava.

With either of these methods, if both kidneys are operated upon, the hypertension will usually be severe, and some will exhibit the malignant syndrome. If only one kidney has been treated, many rats develop satisfactory hypertension. If, however, arterial pressure has not risen after three weeks, the normal kidney may be removed or treated as was the other. When a silk loop has been employed in the initial operation and the pressure has not risen, it is desirable to inspect the kidney before deciding on the next procedure. If it is completely degenerated, or almost so, a second loop should be placed on the opposite renal artery. If, on the contrary, it is normal or even enlarged, but has the appearance of being congested, the partner had best be removed.

Hypertensive rats appear to need supplements to their diet in the form of brewer's yeast (2 per cent of the total diet, strain G, Anghenser-Busch) and liver extract (5 per cent of liver extract No. 343, Lilly). They seem more susceptible to lice and more frequently develop dietary deficiencies than normal rats. In our hands we consider the technique satisfactory if 50 per cent of the animals develop hypertension.

It is of interest that if a rise of arterial pressure to 200 mm. Hg or more develops within a few days of operation, death usually occurs unless a fall in pressure is induced by injection of kidney extract. Under these circumstances they may recover and again become hypertensive on discontinuing the extract, but usually less intensively than before treatment. The time of appearance of hypertension is often very variable, from several days to several months being the rule.

#### DETERMINATION OF SYSTOLIC ARTERIAL PRESSURE IN RATS

The method of Williams, Harrison, and Grollman (1939) has been used. We have found it very satisfactory if a few modifications are introduced. In essence the method consists in determining the pressure at which blood begins to flow into the tail after compression by a cuff, as indicated by a plethysmograph.

*Apparatus.*—The sphygmomanometer cuff (G) in the diagram of Williams, Harrison, and Grollman (1939) was reduced to 2.6 cm. length because a greater length of tail was left for the plethysmograph, and correspondingly the readings were sharper. The tubing should be sufficiently loose so that not more than 10 mm. Hg pressure are required to collapse it. The plethysmograph may be made more sensitive by tying off the rubber sleeve within it at the distal end instead of leaving it open as in the diagram, but we prefer the original method because

the movements of the animal are not unduly magnified. The water in the plethysmograph is warmed by means of an electric light bulb placed close to the syringe *R*. The bore of the rubber tubing leading from the plethysmograph to the water manometer may be as small as  $\frac{3}{32}$  inch in diameter, and the length need not be inconveniently shortened if the wall is thick. The water manometer

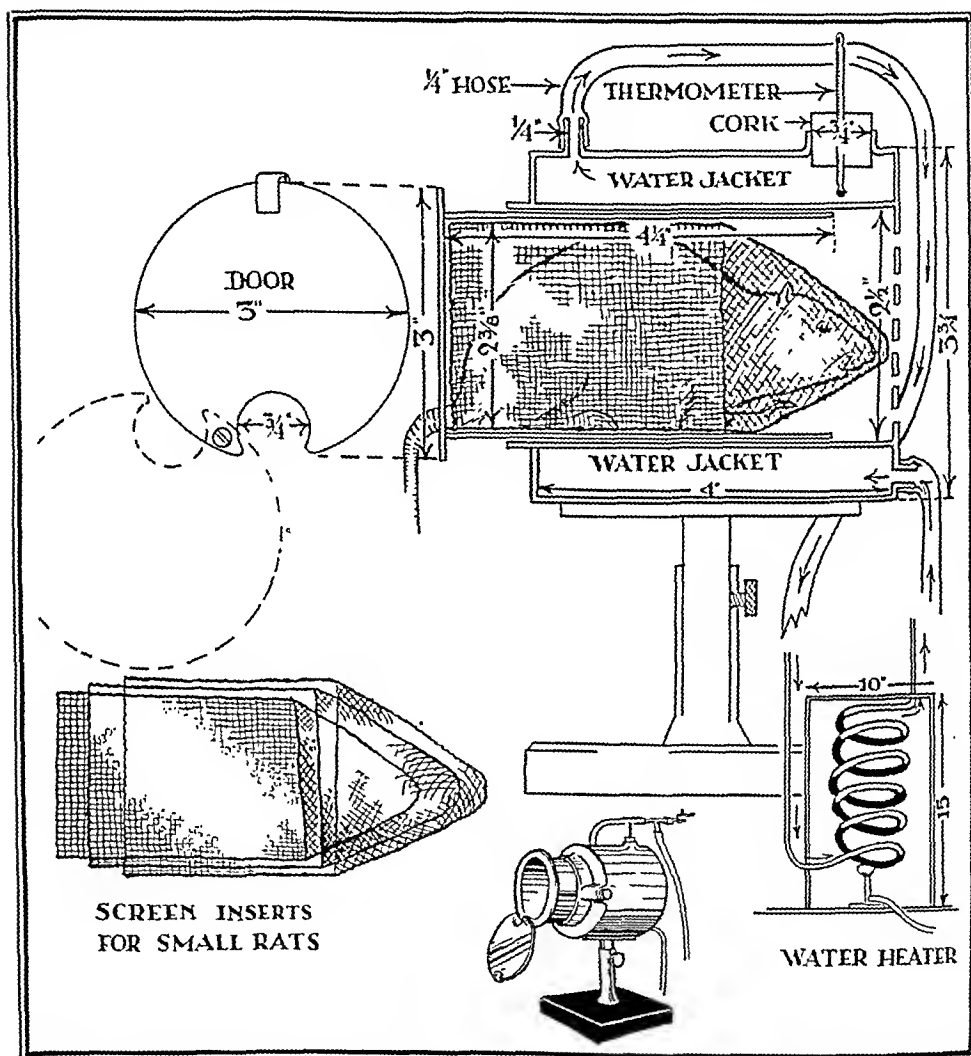


Fig. 1.—Modifications of Williams, Harrison, and Grollman's method for systolic blood pressure in rats.

is easier to read if a little safranin is dissolved in the water filling it. It is important to change the fluid daily to prevent growth of mold which may clog the tubing. Caution set double rubber bags\* seem more convenient than the ordinary sphygmomanometer bulb for reduction of pressure within the system. Instead of using these bulbs for inflating the sphygmomanometer cuff (*G*), air from the laboratory line is employed.

\*Double blast caution bags may be secured from the Davol Rubber Co., Providence, R. I.



The holder (A) for the rat has been adapted (Fig. 1) from that of Chen and Rose (1939) and has been found greatly to facilitate handling of the rats. It is surrounded by a water jacket connected with a copper coil placed in a can which is insulated with asbestos and heated with a micro gas burner. Screen wire adapters (Fig. 1) are used to prevent excess movement by fitting the rat more snugly in the holder.

#### PROCEDURE

The rat is warmed for four to five minutes in a box kept at  $55^{\circ}$  to  $58^{\circ}$  C., placed in the holder (warmed to  $40^{\circ}$  to  $42^{\circ}$  C.), and the tail is inserted into the plethysmograph (warmed to  $40^{\circ}$  C.). Gentle pressure is exerted on the tail by pressing the plunger of the syringe down, the cuff is inflated above the probable systolic pressure, and the two-way cock is turned to connect the plethysmograph with the water manometer. Air is allowed to escape until the water in the manometer begins to rise. If the escape of air is stopped at this point, the mercury in the sphygmomanometer will return to the point at which the water in the manometer began to rise. This return is due to the elastic recoil of the blast bulb. Because of the blast bulb the escape of air can be more rapid and the readings more accurate.

The nearer one is to systolic pressure the slower will be the rise of water in the manometer. A continued rise demonstrates that the change is due to blood entering the tail rather than to the movement of the rat. When the air pressure is lowered to less than venous pressure in the tail, the water in the manometer should fall. Four or five readings are taken on each animal.

#### COMMENT

Comparison of the shorter sphygmomanometer cuff with the longer one shows that readings are sharper and more rapidly taken. Overheating the rats, as evidenced by red ears and paws, overactivity, and sweating, must be avoided. They may suffer a heat stroke and die. By this method of blood pressure determination more uniform and *higher* systolic pressures were obtained on properly warmed animals than on unwarmed or partly warmed ones. Preheating the rats is necessary, as Williams, Harrison, and Grollman state. We find the shorter time at a higher temperature, as suggested by them, somewhat more effective than five to ten minutes at  $40^{\circ}$  to  $42^{\circ}$  C., and we also believe that warming the rat holder is essential. The change in the design of the holder has the very considerable advantage that the rat may be introduced more easily and without excitement. Excited, frightened animals obviously give false values; it is probably not well recognized that defecation while in the holder may raise the pressure 30 mm. Hg, although occasionally a fall will occur. Unusual sounds, especially strange voices, usually affect the readings. Handling the rats by their tails should be scrupulously avoided, as well as giving intramuscular or subcutaneous injections in this region.

The average systolic pressure taken daily for five weeks in five of our normal rats was within the limits of 100 to 120 mm. Lower and much more irregular readings are obtained if the animals and the animal holders are not

warmed. Single readings with the warmed rats were found to correspond with the *average* readings in about one-half of the trials. The other half varied from 20 mm. Hg to occasionally as much as 100 mm. Hg from the average. These results caution against accepting a single reading.

For testing the antipressor potency of kidney extracts we find animals with arterial pressure about 200 mm. Hg most satisfactory. The normal fluctuations of such animals is about 20 mm. Hg; hence a satisfactory extract, when given daily, must reduce the pressure below 150 mm. Hg. After discontinuing extract, the return to the initial blood pressure level usually takes more than a day. At least three hypertensive rats are used for each assay. It is important that the personnel dealing with these animals be sympathetic toward them, and that injections of extracts not be made at or near the time blood pressure readings are taken. If the latter precaution is observed, it will be found much easier to train the animals.

#### SUMMARY

1. Silk perinephritis and constriction of the renal artery by a silk thread both elicit arterial hypertension in rats of a degree sufficient for assay of renal antipressor extracts. The preparation of hypertensive rats by these methods is described.

2. The method of Williams, Harrison, and Grollman for measurement of systolic blood pressure has been modified to increase its effectiveness.

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# A QUANTITATIVE MICROPIPETTE FOR INTRANASAL INOCULATION OF MICE WITH VIRUS SUSPENSIONS

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IT IS standard practice in most laboratories engaged in research on viruses to employ small hypodermic syringes and needles for the intranasal inoculation of mice. When large numbers of animals are being handled, as in our current influenza investigations, we have found that this technique not only requires a very considerable outlay for syringes, but is tiring and somewhat cumbersome in practice.

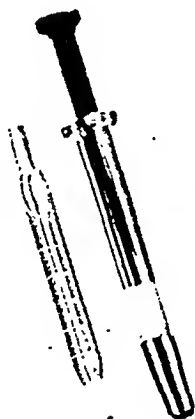


Fig. 1.—Micropipette (left) and syringe fitted with adapter (right).

The apparatus shown in Figs. 1 and 2 was developed to replace the syringe-needle assembly, and has been used with satisfaction during the past several months. The micropipette (left, Fig. 1) is  $3\frac{3}{4}$  inches long and is graduated to deliver five aliquots of 0.05 ml. each. The top of the pipette contains a safety bulb beyond which it tapers to fit in the adapter of the plunger unit. The latter consists of an ungraduated syringe with colored ground-in piston; it is used to generate suction for filling the micropipette and to regulate the delivery of virus suspensions from it.

\*The Unit Personnel consists of Commander A. P. Krueger; Lieutenants (j.g.) W. P. Chesbro, L. E. Rosenberg, and N. S. West; Ensigns A. S. Browne, O. J. Golub, and J. R. Mathews; CPhM, I. L. Shechmeister and T. P. Sislock; PhM 1c, W. L. Axelrod; PhM 2c, E. R. Chisholm and G. B. Saviers; and PhM 3c, H. R. Burkhead and C. R. Webb, Jr.

The opinions advanced in this paper are those of the writers and do not represent the official views of the Navy Department.

Under working conditions the plunger unit is mounted rigidly in a burette clamp, and micropipettes are fitted into the adapter as needed. Usually a fairly large series of pipettes will be employed in succession, using a clean one for each



Fig. 2.—Micropipette assembly in use for conducting intranasal inoculations.

virus-containing suspension to be inoculated. The pipettes cost about one-half as much as a regulation 0.25 ml. hypodermic syringe, and since only one or two plunger units per operator are required for a day's work, the total cost for equipping a laboratory is very reasonable.

# SODIUM PENTOTHAL ANESTHESIA FOR ABDOMINAL SURGERY IN EXPERIMENTAL ANIMALS\*

## INDUCTION BY INTRAVENOUS INJECTION AND MAINTENANCE BY INTERMITTENT INTRAPERITONEAL DRIP

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**S**HORT duration between administration and excretion is a desirable attribute of any anesthetic because it permits more immediate control of the anesthetic level. The adage "better to repeat than to repent" has been impressed upon many surgeons and anesthetists working with various long-action anesthetic agents.

While performing certain experimental abdominal procedures in dogs and rabbits, we developed a maintenance technique with sodium pentothal† which permitted us to dispense with the services of an anesthetist for the entire procedure. If we worked in pairs, the assistant induced the anesthesia by intravenous injection while the operator remained attired in sterile gown and gloves. If we worked alone, the operator induced the anesthesia by intravenous injection while wearing an extra pair of rubber gloves, which were removed before starting the abdominal operation. The instrument tray contained a sterile syringe with a hypodermic needle into which the operator drew sodium pentothal for intraperitoneal administration during the course of the operation.

For rabbits we used 1.25 and 1 per cent solutions of sodium pentothal. The latter was more satisfactory. The animals weighed approximately 4 pounds. They were placed in a box with the head protruding, and 2 to 3 c.c. of 1 per cent solution were administered in the lateral marginal vein of the ear. With this amount, corneal reflexes still remain. Five cubic centimeters of 1 per cent solution are lethal. The animals were then strapped on the operating table and the procedure was started. If a minimal induction dose was used, the animal might be restless during the opening of the peritoneum. A few drops of 1 per cent solution administered from the syringe on the tray would stop this. After the peritoneum was opened the entire length of the incision, little difficulty was encountered. Maintenance of anesthesia was done by dropping intermittently a small amount of sodium pentothal into the open abdomen, guided by restlessness of the animal and the character of its respirations. Shallowness of respiratory exchange indicated too great depth. An average

\*From the Foundation for Clinical and Surgical Research, Dr. John O. Bower, Director.  
†A product of the Abbott Laboratories.  
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anesthesia was performed for wedge gastric resection wherein 2 c.c. of 1 per cent solution were given intravenously for induction, and 1.4 c.c. by intraperitoneal drip for maintenance. The operative procedure lasted one-half hour. More than 50 rabbits have been operated on with the above technique.

For dogs we used 2 and 2.5 per cent solutions. The animals were strapped on the operating table, and the induction dose was given into a superficial vein of the forepaw or the hind leg. The amount necessary varied with the size and vigor of the animal. Morphine and atropine preoperatively were of great advantage. Dogs weighing less than 20 pounds received morphine sulfate gr.  $\frac{1}{8}$  and atropine sulfate gr.  $\frac{1}{150}$ ; those weighing more than 20 pounds received morphine sulfate gr.  $\frac{1}{4}$  and atropine sulfate gr.  $\frac{1}{100}$ . This preoperative sedation was given hypodermically forty-five minutes before. Little difficulty was found in opening the peritoneum. Anesthesia was maintained by intermittent intraperitoneal drip. Anesthetic level was determined by respirations and restlessness, and the status of corneal reflex was obtained by touching the cornea with a piece of gauze which was then discarded. A representative anesthesia was performed in a 60 pound dog, to which 14 c.c. of 2.5 per cent solution were given intravenously for induction and 12 c.c. were given intraperitoneally for maintenance during a procedure on the biliary ducts, lasting one hour. More than 25 dogs have been operated upon with this anesthetic technique.

2008 WALNUT STREET

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## THE SEDIMENTATION RATE IN HODGKIN'S DISEASE\*

BOWMAN WISE, M.D., DURHAM, N. C.

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IT IS generally recognized that the erythrocyte sedimentation rate usually is significantly elevated in Hodgkin's disease, but that it is of no aid in the differential diagnosis of this disease. However, the relationship of the sedimentation rate to phases of activity, or relative quiescence, of the disease has not been studied. The present study was undertaken to determine whether the sedimentation rate might be of aid in determining the state of activity of the Hodgkin's disease process, thereby furnishing indications for treatment, especially during intervals in which the patient is symptom free and has no objective evidence of activity of the disease.

### MATERIAL AND METHOD OF STUDY

The sedimentation rate of the red blood corpuscles has been determined by the Wintrobe method.<sup>1</sup>

The data reported here have been accumulated from patients in Duke Hospital in whom the diagnosis of Hodgkin's disease has been established histo-

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pathologically. Sedimentation rates have been determined at the time of the initial visit and, whenever possible, at intervals throughout the period of observation. Because patients frequently leave the hospital or out-patient clinic promptly upon the completion of roentgen therapy, it has not been possible to make observations of the immediate effect of such therapy on the sedimentation rate.

### RESULTS

Single determinations of the sedimentation rate were made on 17 patients suffering from Hodgkin's disease, the results of which are given in Table I. They show a significant elevation of the sedimentation rate in Hodgkin's disease and an absence of any correlation with the duration of the disease or therapy. In all these patients, because of the existence of fever, obvious progressive lymph node enlargement, or evidence of progressive visceral or osseous involvement, the disease process was considered to be active at the time the sedimentation rates were determined.

Thirteen patients afforded an opportunity to make repeated determinations of the sedimentation rate during the course of the disease. The case histories of the majority of these patients have been reported in detail elsewhere<sup>2</sup> and are summarized briefly here in the numerical sequence in which the patients came under observation.

TABLE I  
BLOOD SEDIMENTATION RATES OF 17 PATIENTS SUFFERING FROM HODGKIN'S DISEASE

| PATIENT | RACE | SEX | AGE | CORRECTED<br>SEDIMENTA-<br>TION RATE<br>(MM./HR.) | DURATION OF<br>DISEASE BY<br>HISTORY | PREVIOUS THERAPY                        |
|---------|------|-----|-----|---|--------------------------------------|---|
| J. O.   | W    | M   | 20  | 43  | 2½ years                             | Roentgen therapy                        |
| L. S.   | W    | F   | 44  | 44  | 2 years                              | Roentgen therapy                        |
| V. W.   | W    | F   | 26  | 40  | 8 to 9 years                         | Roentgen therapy                        |
| M. H.   | W    | F   | 24  | 29  | 20 months                            | None                                    |
| S. S.   | W    | M   | 38  | 34  | 21 months                            | Roentgen therapy                        |
| E. W.   | W    | M   | 28  | 39  | 8 years                              | Roentgen therapy                        |
| V. H.   | W    | F   | 21  | 42  | 6 months                             | None                                    |
| P. T.   | W    | F   | 29  | 39  | 22 months                            | Sulfapyridine and roent-<br>gen therapy |
| C. S.   | W    | M   | 38  | 37  | 4 years                              | Roentgen therapy                        |
| J. C.   | W    | M   | 30  | 32  | 13 months                            | Roentgen therapy                        |
| S. M.   | W    | F   | 43  | 38  | 3 months                             | Roentgen therapy                        |
| E. M.   | W    | M   | 26  | 32  | 9 months                             | None                                    |
| P. C.   | W    | F   | 25  | 46  | 8 months                             | None                                    |
| K. G.   | W    | M   | 17  | 40  | 5 years                              | Roentgen therapy                        |
| F. T.   | W    | M   | 30  | 40  | 2 months                             | None                                    |
| S. G.   | W    | M   | 38  | 32  | 8 months                             | Roentgen therapy                        |
| W. D.   | W    | M   | 31  | 25  | 2 years                              | Roentgen therapy                        |

Case 1 is of particular interest because of the demonstration of normal or nearly normal sedimentation rates during periods in which there was no evidence of activity of the disease. In this patient elevation of the sedimentation rate from a previous normal level preceded by six weeks any definite evidence of recurrent activity of the disease process.

## CASE REPORTS

CASE 1.—R. P., a 24-year-old white man, entered the hospital on Oct. 26, 1938, complaining of right cervical lymph node enlargement of one year's duration, which had been treated by excision and superficial radium therapy. At the time of admission a lymph node, 3 to 4 cm. in diameter, and several smaller nodes were palpable in the right cervical region. *Brucella suis* was isolated from a right cervical lymph node which had the characteristic histopathologic appearance of Hodgkin's disease.

| DATE     | CORRECTED SEDI-<br>MENTATION RATE<br>MM./HR. | SUBSEQUENT COURSE  |
|----------|--|--|
| 10/27/38 | 5  | Regression of right cervical lymph node enlargement under treatment with sulfanilamide and serum from a person considered immune to brucella infection   |
| 7/31/39  | 4  | No evidence of activity of the Hodgkin's disease process   |
| 9/23/39  | 8  | Questionable slight recurrent enlargement of right cervical lymph nodes following acute pharyngitis two weeks previously   |
| 11/27/39 | 20   | No evidence of activity  |
| 1/29/40  | 34   | Recurrent right cervical lymph node enlargement regressing slightly under sulfanilamide and roentgen therapy   |
| 4/15/40  | 34   | Condition unchanged  |
| 6/10/40  | 25   | Cough of two weeks' duration; mediastinal mass demonstrable in chest roentgenogram; marked diminution of cough during treatment with sulfathiazole, but size of mass unchanged; roentgen therapy instituted to mediastinum |
| 8/ 6/40  | 34   | Chills and fever of three weeks' duration; complete atelectasis of left lung from pressure of mediastinal mass; roentgen therapy   |
| 10/ 8/40 | 36   | Fever; weight loss; persistent atelectasis of left lung; liver and spleen palpable   |
| 10/ 8/40 | 34   | Condition unchanged under further roentgen therapy   |
| 12/ 2/40 | 15   | Fever, marked weight loss and anemia; reexpansion of lung; liver and spleen increase in size; questionable infiltration of lumbar spine; no improvement under roentgen therapy; death on Feb. 7, 1941                      |

Cases 2, 7, and 11 show a tendency of the elevated sedimentation rate to remain at a constant level during a period of months, even though temporary improvement in the patient's condition or relative inactivity of the disease may be apparent.

CASE 2.—M. W., a 5-year-old negro boy, was brought to the hospital on Nov. 16, 1938, with an enlargement of his neck of ten months' duration. It was not possible to admit the patient to the hospital until Jan. 26, 1939, at which time examination revealed marked enlargement of the left cervical and left axillary and inguinal nodes. A chest roentgenogram showed a mediastinal mass. Lymph node biopsy confirmed the clinical diagnosis of Hodgkin's disease. Under treatment with sulfapyridine there was a marked decrease in the size of the enlarged superficial lymph nodes, and roentgenographic examination showed diminution in the size of the mediastinal mass. The patient was discharged from the hospital on March 3, 1939. In May, 1939, he was readmitted to the hospital with an enlargement of the left inguinal and femoral lymph nodes, but there was no recurrent enlargement of other superficial lymph nodes, and a chest roentgenogram showed complete absence of the mediastinal



mass noted before. His temperature reached 39° C. *Brucella melitensis* was isolated from a blood culture. Following treatment with sulfapyridine and bovine antimelitensis serum, the patient became afebrile, and the enlarged lymph nodes had decreased in size by the time of discharge on June 4, 1939. The patient's subsequent course is tabulated below:

| DATE     | CORRECTED SEDI-<br>MENTATION RATE<br>MM./HR. | SUBSEQUENT COURSE  |
|----------|--|--|
| 9/ 8/39  | 28   | Fever and recurrent left cervical, axillary and inguinal lymph node enlargement of one week's duration, subsiding under treatment with sulfapyridine   |
| 2/ 5/40  | 28   | History of intermittent brief episodes of fever; transient left cervical enlargement one week previously   |
| 6/24/40  | 30   | Condition unchanged; no demonstrable recurrence of lymph node enlargement  |
| 12/12/40 | 21   | Intermittent enlargement of superficial lymph nodes accompanied by fever of one month's duration; spleen palpable  |
| 6/13/41  | 22   | Marked enlargement of cervical, axillary, and inguinal lymph nodes; fever of one week's duration; spleen and liver palpable; prompt regression of lymph node enlargement under sulfathiazole |
| 6/23/41  | 26   |  |
| 6/27/41  | 15   |  |

CASE 7.—M. T., a 31-year-old white woman, experienced the onset of persistent generalized itching in November, 1936, along with enlargement of left cervical and left axillary nodes which fluctuated in size, but there had been recurrent enlargement of these nodes for two months before admission to the hospital on March 8, 1939. Lymph node biopsy confirmed the diagnosis of Hodgkin's disease. Her subsequent course is given below:

| DATE     | CORRECTED SEDI-<br>MENTATION RATE<br>MM./HR. | SUBSEQUENT COURSE   |
|----------|--|---|
| 3/ 8/39  | 38   | Left cervical and axillary lymph node enlargement; severe pruritus; lessened pruritus and decrease in the size of the enlarged lymph nodes under treatment with sulfapyridine                                       |
| 5/ 7/39  | 38   | Recurrence of lymph node enlargement with enlargement of right cervical and axillary nodes, and small areas of density shown in the right lung by roentgenogram; stomatitis; fever; improvement under sulfapyridine |
| 7/18/39  | 28   | Recurrent superficial lymph node enlargement; cough and extensive mediastinal and hilar enlargement demonstrable roentgenographically; intensified pruritus; improvement under roentgen therapy                     |
| 9/11/39  | 44   | Further roentgen therapy; cough relieved; pruritus unchanged  |
| 10/18/39 | 43   | Slight enlargement of left cervical lymph nodes; increasing weakness  |
| 11/23/39 | 44   | Fever; extension of pulmonary parenchymal involvement; progressive weakness, weight loss, and anemia; further roentgen therapy  |
| 2/12/40  | 37   | Afebrile; further superficial lymph node enlargement, including inguinal nodes; decrease in size of pulmonary lesions by roentgen examination   |
| 3/14/40  | 42   | Fever and progressive cachexia; vague abdominal discomfort; further roentgen therapy  |
| 9/12/40  | 18   | Continued fever and progressive cachexia terminating in death on Sept. 20, 1940   |

CASE 11.—A. A., a 40-year-old woman, entered the hospital on June 27, 1939, complaining of weakness, loss of weight, and enlargement of the neck of seven months' duration. Above the suprasternal notch and in the right cervical region were numerous enlarged lymph nodes constituting a mass 5 by 4 cm. The spleen was palpable. A chest roentgenogram showed a large mediastinal mass. Lymph node biopsy confirmed the clinical impression of Hodgkin's disease, and from the biopsied node and from the blood *Brucella suis* was isolated. Her subsequent history is summarized below:

| DATE     | CORRECTED SEDI-<br>MENTATION RATE<br>MM./HR. | SUBSEQUENT COURSE  |
|----------|--|--|
| 6/27/39  | 34   | Moderate decrease in cervical and mediastinal lymph node enlargement under roentgen therapy          |
| 9/ 8/39  | 34   | Weight gain of 11 pounds in interval; no recurrent lymph node enlargement; marked fatigability       |
| 12/22/39 | 34   | Slight recurrent enlargement of cervical nodes; enlargement of left inguinal nodes; roentgen therapy |

Cases 6, 17, and 18 show a definite fall in the sedimentation rate following sulfonamide and/or roentgen therapy.

CASE 6.—C. I., a 40-year-old white woman, in June, 1938, noted gradual enlargement of the superficial lymph nodes accompanied by fever and marked fatigability. In January, 1939, mediastinal enlargement was demonstrable, and the liver and spleen were palpable. Lymph node biopsy established the diagnosis of Hodgkin's disease. Under roentgen therapy her fever subsided, the lymph node enlargement disappeared, and the liver and spleen were no longer palpable. Her subsequent course as is known is given below:

| DATE    | CORRECTED SEDI-<br>MENTATION RATE<br>MM./HR. | SUBSEQUENT COURSE  |
|---------|--|--|
| 3/ 1/39 | 26   | Initial visit two weeks after completion of roentgen therapy   |
| 6/ 6/39 | 36   | Fever and slight enlargement of a right cervical node; spleen palpable; <i>Brucella melitensis</i> isolated from blood cultures. Prompt subsidence of symptoms and splenic enlargement under treatment with sulfapyridine and bovine antimeliten-sis serum |
| 7/20/39 | 19   | Entirely symptom free  |

CASE 17.—G. R., a 16-year-old white boy, entered the hospital on March 8, 1940, complaining of superficial lymph node enlargement of two years' duration. Lymph node biopsy in August, 1939, established the diagnosis of Hodgkin's disease, and he was subsequently given roentgen therapy, with reduction in the size of the enlarged nodes. At the time of admission to the hospital there were daily elevations of temperature to 39° C., a few small firm lymph nodes were generally palpable, and the spleen, which was firm and nontender, extended 10 cm. below the costal margin. A chest roentgenogram was not remarkable.

| DATE   | CORRECTED SEDI-<br>MENTATION RATE<br>MM./HR. | SUBSEQUENT COURSE   |
|--------|--|---|
| 3/4/40 | 18   | Initial visit   |
| 3/8/40 | 18   | Temperature fell under sulfathiazole therapy, but the patient did not become afebrile until roentgen therapy was instituted to splenic area; spleen ceased to be palpable |
| 4/5/40 | 7  |   |

CASE 18.—J. Q., a 23-year-old white man, was referred to the hospital on April 20, 1940, for study. His illness began in December, 1934, with generalized pruritus, which persisted until the summer of 1937. In May, 1937, a slight enlargement of the cervical and axillary lymph nodes was noted, and the histopathologic findings in an excised node were typical of Hodgkin's disease. A chest roentgenogram taken at this time showed hilar enlargement. Roentgen therapy was instituted in August, 1937, since which time the patient had been almost symptom free. In September, 1939, a few small nodes were noted in the axillae. At the time of his visit to the hospital he felt entirely well, but he had noted an elevation of temperature to 99.1° F. each evening of the preceding week. Physical examination revealed two very small palpable right axillary nodes. His subsequent course during the period of observation is given below:

| DATE    | CORRECTED SEDIMENTATION RATE<br>MM./HR. | SUBSEQUENT COURSE  |
|---------|---|--|
| 4/20/40 | 47                                      | Initial visit  |
| 6/ 7/40 | 20                                      | Condition unchanged. Slight daily elevation of temperature continued |
| 6/17/40 | 14                                      | Patient received sulfathiazole from 6/11/40 to 6/26/40               |
| 6/19/40 | 11                                      |  |

#### DISCUSSION

The finding of a normal or low erythrocyte sedimentation rate during a period of apparent quiescence of the Hodgkin's disease, suggests that this procedure may have a distinct usefulness in following the course of the Hodgkin's disease process. In particular, the finding of an elevated sedimentation rate may aid in deciding when further therapy is advisable.

It has not been possible as yet in this study to determine the immediate effect of roentgen therapy upon the sedimentation rate in Hodgkin's disease. Patients in whom the progress of the disease process has been relatively constant and rapid, and who from time to time have received roentgen therapy, have maintained elevated sedimentation rates. However, these determinations of the sedimentation rate have not been made at sufficiently close intervals to furnish information regarding the effect of roentgen therapy during the period when its effect is clinically most obvious.

In patients suffering from Hodgkin's disease, treatment with sulfonamide drugs has frequently been followed by regression of lymph node enlargement, disappearance of fever, and improvement in the patient's general condition. Although the number of observations is few, a fall in the sedimentation rate has attended the good results of sulfonamide therapy. Only further investigation and experience can determine the proper place of the sulfonamide drugs in the treatment of Hodgkin's disease, but repetition of the sedimentation rate at frequent intervals during the treatment period, regardless of the type of therapy employed, may be expected to aid in the decision as to the amount and extent of treatment advisable.

Attention should be called to a fall in the sedimentation rate exhibited by two patients of this group, Cases 1 and 7, during the terminal stage of the disease. Wintrobe has stated that the sedimentation rate may be normal, or fall toward normal, in the markedly cachectic stage of tuberculosis, and that corrections of

the sedimentation rate in the presence of severe anemia may be misleading.<sup>3</sup> Both patients, mentioned above, exhibited severe anemia and cachexia, to which the fall of the sedimentation rates seems attributable.

#### SUMMARY

The sedimentation rate has been studied during the course of Hodgkin's disease. Although usually elevated in this disease, the sedimentation rate may be normal during periods of relative inactivity of the disease process. The usefulness of the sedimentation rate in following the course of Hodgkin's disease and as a guide in therapy is suggested.

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## A MERCURY VACUUM RELEASE\*

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### FOR ULTRAFILTRATION AND OTHER EVACUATED SYSTEMS

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**I**N CONNECTION with many laboratory experiments it is desirable to maintain a vacuum of a given level. While a simple mercury trap is adequate when the vacuum desired is only a few millimeters of mercury, for a higher vacuum, bubbling and loss of mercury is troublesome unless a large diameter tube is used, in which event a great deal of mercury is required. The device we describe is easily constructed, and is capable of maintaining the vacuum in a given system with a variation of only a few millimeters.

A 120 mm. T-tube is connected to the upper end of a 50 c.c. burette; through this, held by a piece of pressure tubing, is run a length of 5 mm. glass tubing, of such dimensions that when bent the upper end extends about 2 inches below the mouth of the burette, and the lower end about 1 inch above the tip. By a heavy clamp, not shown in the diagram, placed near the upper end of the burette, the whole device is suspended in a bottle containing mercury. When connected to the system to be held at a given vacuum, the mercury rises in both tubes. When the level of the mercury drops below the tip of the glass tube, the mercury in this tube is drawn over, returning through the burette to the bottle,

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and allowing air to enter the system. By adjusting the depth which the tubes penetrate into the mercury, in conjunction with a manometer, the device may be set so that air enters the system at any given vacuum level, and is by-passed to the pump, making certain that the vacuum does not exceed the desired level.

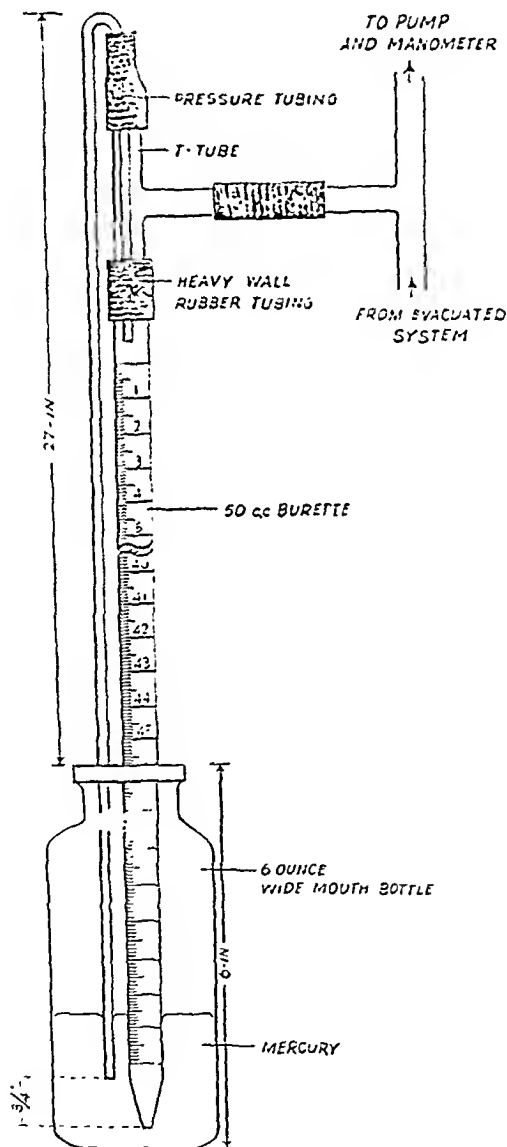


FIG. 1.

Variation in the internal pressure in the evacuated system may be minimized by drawing out the lower end of the tube to a small tip.

We have used this for maintaining vacua of from 15 to 360 mm. of mercury and have found it satisfactory for ultrafiltration and for holding animals at varying fractions of atmospheric pressure.

## CHEMICAL

### EFFECT OF STANDING ON CHOLESTEROL AND CHOLESTEROL ESTER VALUES IN HUMAN BLOOD\*

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**S**TUDIES on cholesterolysis, cholesterogenesis,† cholesterol ester hydrolysis, and esterification of the different parts of human blood (blood serum, blood plasma, whole blood) have presented varied and conflicting results.

Schultz,<sup>1</sup> Thannhauser,<sup>2</sup> Bloor and Knudson,<sup>3</sup> found no change in the cholesterol values on incubation of human blood while Cytronberg<sup>4</sup> and Sperry<sup>5</sup> report marked hydrolysis and marked esterification. Schube<sup>6</sup> states that blood cholesterol may change in vivo from week to week from 0 to 73 mg. He made a study<sup>7</sup> of 21 normal bloods and showed that after different time intervals and at different temperatures, there was a variation from 15 points above to 49 points below the original cholesterol values.

Inasmuch as the per cent of cholesterol esters in the total blood cholesterol is of diagnostic significance in cases of liver damage and jaundice,<sup>3, 8-12</sup> the question arose in our laboratory as to whether it was safe to allow the blood to stand any length of time before analyzing it for cholesterol and cholesterol esters, or whether these determinations should be made immediately after the blood was drawn.

To answer these questions, a comparative study of cholesterolysis, cholesterogenesis, and cholesterol ester hydrolysis and esterification on (a) oxalated blood, (b) plasma from oxalated blood, and (c) blood serum was made.

#### PROCEDURE

The bloods were selected at random from those of patients and blood donors‡ in the Cook County Hospital. Duplicate determinations for both total cholesterol and cholesterol esters were made immediately after the blood was drawn and after the specimens had stood for twenty-four hours.

The plasma and serum were separated from the cells by centrifugation and placed in cork-stoppered containers. The whole oxalated blood was kept in stoppered tubes. For the "immediate" analyses, aliquots were taken soon after the bloods were drawn, and alcohol ether filtrates were made at once (as directed below) and set aside in glass-stoppered bottles until the twenty-four-hour filtrates were ready. For the "twenty-four-hour" analyses the specimens were tightly stoppered and allowed to stand for twenty-four hours at room temperature or in the incubator at 37° C.; aliquots were then taken and alcohol-

\*From the Department of Biochemistry, Cook County Hospital, Chicago.

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†"Cholesterolysis, cholesterogenesis" used in the same sense as the terms "glycolysis and glycogenesis."

‡Blood from normal donors was obtained through the courtesy of Dr. E. H. Schirmer, Director, Blood Bank, Cook County Hospital.

ether filtrates were made. Total cholesterol and cholesterol ester determinations were made simultaneously to rule out additional time factors.

*Total Cholesterol* (Bloor,<sup>13</sup> Sachett<sup>14</sup> method slightly modified).—Three cubic centimeters of oxalated blood, plasma, or serum are transferred into a 100 c.c. volumetric flask, using an Oswald-Folin pipette;\* 3:1 alcohol-ether mixture is added through a funnel while agitating the flask.† Make up to volume, mix well, and filter. If the filtrate is tinted yellow (icteric blood), shake with Lloyd's reagent and refilter. Use aliquots of the clear filtrate for duplicate determinations‡ of cholesterol or cholesterol esters.

TABLE I

TOTAL CHOLESTEROL (MG./100 C.C.)  
(RESULTS OF FIRST 20 CASES FROM A SERIES OF 78\* CASES)

| WHOLE BLOOD      |                |            | PLASMA           |                |            | SERUM            |                |            |
|------------------|----------------|------------|------------------|----------------|------------|------------------|----------------|------------|
| IMMEDIATE VALUES | 24-HOUR VALUES | DIFFERENCE | IMMEDIATE VALUES | 24-HOUR VALUES | DIFFERENCE | IMMEDIATE VALUES | 24-HOUR VALUES | DIFFERENCE |
| 184              | 183            | -1.0       | 115              | 120            | +5.0       | 172              | 171            | -1.0       |
| 163.5            | 157.5          | -6.0       | 193              | 197.5          | +4.5       | 168              | 168            | 0.0        |
| 172.5            | 170            | -2.5       | 148.5            | 147            | -1.5       | 279              | 279            | 0.0        |
| 170.5            | 168.5          | -2.0       | 131.5            | 128.5          | -3.0       | 241              | 240            | -1.0       |
| 185.5            | 186            | +0.5       | 185.5            | 186.5          | -1.0       | 243              | 239.5          | -3.5       |
| 151.5            | 150.5          | -1.0       | 186              | 186.5          | +0.5       | 202              | 198.5          | -3.5       |
| 143              | 142            | -1.0       | 126.5            | 125.5          | -1.0       | 305.5            | 310            | +4.5       |
| 190              | 190            | 0.0        | 225              | 226            | -1.0       | 266              | 267            | +1.0       |
| 154.5            | 155.5          | +1.0       | 176              | 177            | -1.0       | 206              | 204.5          | -1.5       |
| 170.5            | 170.5          | 0.0        | 159.5            | 157            | -2.5       | 181.5            | 177            | -4.5       |
| 200              | 199            | -1.0       | 181.5            | 182.5          | -1.0       | 223.5            | 219            | -4.5       |
| 150              | 146            | -4.0       | 334              | 331            | -3.0       | 213              | 209            | -4.0       |
| 247              | 244            | -3.0       | 155.5            | 155            | -0.5       | 256              | 254            | -2.0       |
| 214.5            | 212            | -2.5       | 212.5            | 210            | -2.5       | 147.5            | 149.5          | +2.0       |
| 152              | 152.5          | +0.5       | 195              | 195.5          | +0.5       | 207.5            | 209            | +1.5       |
| 148.5            | 146            | -2.5       | 147              | 148            | +1.0       | 157.5            | 156.5          | -1.0       |
| 222.5            | 220            | -2.5       | 168.5            | 169            | +0.5       | 193              | 196            | +3.0       |
| 250              | 250            | 0.0        | 213              | 213            | 0.0        | 250              | 252.5          | +2.5       |
| 220              | 218.5          | -1.5       | 151              | 152.5          | +1.5       | 240.5            | 242.5          | +2.0       |
| 218              | 213            | -5.0       | 168              | 170.5          | +2.5       | 169              | 167            | -2.0       |

\*Complete protocols available on request.

*Cholesterol Esters.*—Cholesterol is precipitated from the filtrate with digitonin. A quantitative separation of the cholesterol esters from the cholesterol digitonide is made by extraction with petroleum ether (b.p. 60° C.) as follows:

Add 15 c.c. of petroleum ether to the residue from 25 c.c. of evaporated filtrate. The mixture is thoroughly scraped from the sides and bottom of the beaker and triturated. Cover the beaker with a watch glass and boil on a water bath over an electric plate, rotating constantly to avoid bumping, until about one-half of the liquid has evaporated. Dry the beaker on the outside, and when boiling ceases, decant the solution with the aid of a stirring rod through a small funnel plugged with a small piece of cotton. The treatment with petroleum ether is repeated three or four times in order to insure quantitative extraction of the cholesterol esters. *The filtrate must be absolutely clear.* Any trace of turbidity will give high results. Evaporate the combined extracts to dryness, and proceed as under cholesterol with chloroform extraction.

\*Twenty milligrams of potassium oxalate for 10 c.c. of blood.

†A fine granular precipitate is obtained, no lumps.

‡Not more than two or three sets of duplicates were set up at any one time.

Using the foregoing technique, we were always able to get like results with duplicate and triplicate samples.

Table I shows the difference between the cholesterol values of whole blood, serum, or plasma tested immediately after drawing, and again in twenty-four hours. This difference is so slight that it is of the same magnitude as the limits of experimental error. This indicates, contrary to the contention of Sehube,<sup>7</sup> that the total cholesterol does not change significantly, if at all, when oxalated whole blood, plasma, or serum is allowed to stand at room temperature for twenty-four hours. It appears, therefore, that a specimen for total cholesterol determination may safely be analyzed within twenty-four hours.

On the other hand, Table II shows that the values of cholesterol esters definitely change as the specimens stand. Of the 58 specimens studied, 25 showed a significant increase in esters or an esterification on standing, ranging from 11 to 74 mg. per 100 c.c.; 9 showed a significant decrease, or a hydrolysis, on standing, ranging from -5.5 to -19 mg. per 100 c.c.; the remaining 24 specimens showed little change.

TABLE II  
CHOLESTEROL ESTER (MG./100 C.C.)  
(RESULTS OF 20 CASES FROM A SERIES OF 58\* CASES)  
(WHOLE BLOOD†)

| TOTAL<br>CHOLESTEROL | CHOLESTEROL ESTER |                |            |
|----------------------|-------------------|----------------|------------|
|                      | IMMEDIATE VALUES  | 24-HOUR VALUES | DIFFERENCE |
| 167                  | 83.5              | 84             | + 0.5      |
| 219                  | 65.5              | 100            | +34.5      |
| 128                  | 53                | 127            | +74.0      |
| 156                  | 32.5              | 91.5           | + 9.0      |
| 144                  | 61.5              | 81             | +19.5      |
| 181                  | 118.5             | 155            | +36.5      |
| 203                  | 160               | 172            | +12.0      |
| 225                  | 80                | 106.5          | +26.5      |
| 154                  | 60.5              | 60             | - 0.5      |
| 183                  | 64                | 101            | +37.0      |
| 186                  | 126.5             | 126            | - 0.5      |
| 214                  | 120.5             | 169            | +48.5      |
| 218                  | 126               | 126            | 0.0        |
| 175                  | 101.5             | 90             | -11.5      |
| 233                  | 65.5              | 66             | + 0.5      |
| 169                  | 98                | 95.5           | - 2.5      |
| 138                  | 64                | 64             | 0.0        |
| 155                  | 103.5             | 107            | + 3.5      |
| 163                  | 79                | 90.5           | +11.5      |
| 164                  | 92                | 93.5           | + 1.5      |

\*Complete protocols available on request.

†Similar results were obtained with plasma and with serum.

These results confirm the presence of esterase activity in human bloods.<sup>5</sup> The presence and extent of that activity are unpredictable and varied and, therefore, must be evaluated for each specimen.

Since the percentage of cholesterol esters in the total cholesterol is used clinically as a diagnostic aid in liver damage, the determination, to be of value, must be made on freshly drawn blood.

The original value for cholesterol or cholesterol esters does not indicate the degree or direction of the possible changes on standing.



To establish whether the "enzymatic" activity present in some bloods and absent in others is transitory or is relatively constant, second determinations were made on the same patients a week later. The presence of the activity was shown to be relatively constant.

### CONCLUSIONS

1. The total cholesterol content of human blood, plasma, or serum does not change on standing.
2. The value of cholesterol esters in human blood, plasma, or serum may or may not change on standing.
3. Cholesterol determinations on whole blood, plasma, or serum need not be performed immediately after the blood is drawn. Cholesterol ester determinations must be made immediately.
4. The presence of an "esterase activity" producing an hydrolysis of cholesterol esters in some specimens and an esterification of cholesterol in others is confirmed. The nature of this activity, and why it is found in some bloods and not in others, is being investigated.

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## THE QUANTITATIVE DETERMINATION OF BLOOD IN HUMAN FECES\*

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### INTRODUCTION

ALTHOUGH there are many references in medical literature dealing with problems associated with the detection and clinical significance of occult blood in the stomach and intestinal contents of patients suffering from hemorrhage within the gastrointestinal tract, there are very few papers approaching the subject from the standpoint of quantitative measurement. Abrahams<sup>1</sup> and Bramkamp<sup>2</sup> reported experiments designed to ascertain the smallest quantity of blood which after ingestion would produce a stool giving a positive reaction to the benzidine test for occult blood. Later Daniel and Egan,<sup>3</sup> working with larger quantities of blood, found that 50 to 80 c.c. of blood must be ingested by adults on a milk and cream diet in order to produce a so-called "farry" stool. Although van Eek<sup>4</sup> published a method for the quantitative determination of blood in human feces, careful search of the literature has failed to produce any evidence of its practical application in clinical medicine. In a recent paper<sup>2</sup> Andrews and Brooks outlined a procedure for the quantitative determination of blood in the feces of sheep by means of the Evelyn Photoelectric Colorimeter, incorporating the technique of Bing and Baker<sup>4</sup> for the hemoglobin determination. Since we were of the opinion that one of the more important uses of this method lies in its application to the quantitative study of hemorrhage within the human gastrointestinal tract, we carried out the following experiments.

### MATERIALS AND METHODS

The materials and reagents necessary for the work, as outlined in the experiments are as follows:

1. Blood-free human feces.
2. 50 c.c. round-bottomed heavy glass centrifuge tubes.
3. Blood of known hemoglobin content.
4. Accurately calibrated pipettes of 1, 2, 4, 5, 10, 15, and 20 c.c. capacity.
5. Glacial acetic acid.
6. 50 c.c. volumetric flasks.
7. 10 c.c. volumetric flasks.

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8. Purified benzidine reagent of Bing.<sup>10</sup>
9. Test tubes for the Evelyn photoelectric colorimeter.
10. 3 per cent hydrogen peroxide.
11. Paraffined corks to fit the colorimeter tubes.
12. No. 660 red filter for the colorimeter.

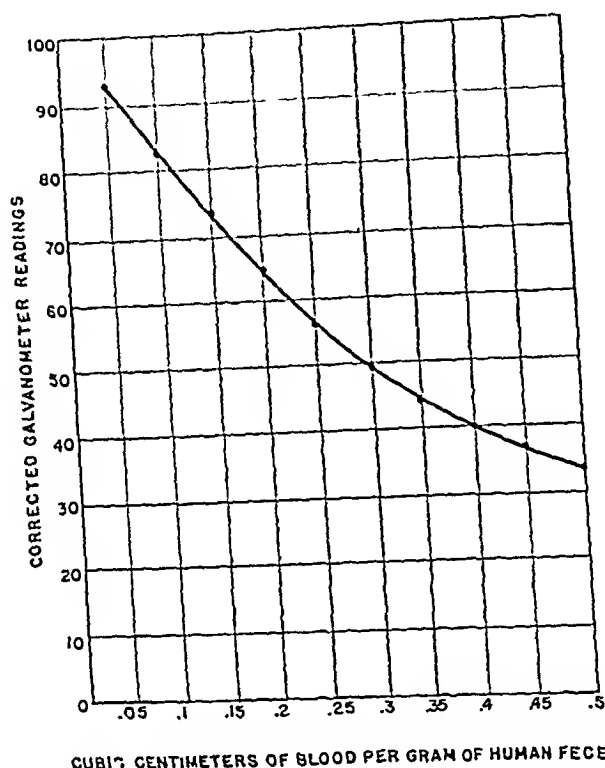


Fig. 1.—Curve showing the corrected galvanometer readings obtained from the analysis of 1 Gm. portions of human feces to which had been added the indicated volumes of calf blood containing 9 Gm. of hemoglobin per 100 c.c.

#### PROCEDURE

We first placed ourselves on a meat-free diet in order that the feces we passed would not contain ingested blood pigment. To 1 Gm. portions of blood-free human feces collected on the third day, artificial mixtures of known amounts of calf blood, containing 9 Gm. of hemoglobin per 100 c.c. were added, and a graph (Fig. 1) relating the quantity of blood to the colorimetric measurement

\*Dissolve the benzidine reagent in 200 c.c. of 95 per cent ethyl alcohol with the aid of gentle heat. Add 1 Gm. of blood charcoal (extracted with hydrochloric acid and washed with water) to the filtrate, stir, and maintain at a temperature of  $50^{\circ} \pm 10^{\circ}$  C. for fifteen minutes. Filter while warm and wash charcoal with 10 c.c. of warm ethyl alcohol. Repeat treatment with charcoal until filtrate is almost colorless. To filtrate add 135 c.c. of distilled water, or sufficient water to make alcohol 50 to 60 per cent by volume, and warm if necessary to obtain a clear solution. Allow solution to stand in the refrigerator for twenty-four to forty-eight hours to crystallize the benzidine. Filter on a Buchner funnel with suction, wash with cold 50 per cent alcohol, and continue suction until crystals are dry. Preserve in a bottle protected from light.

Prepare the reagent by dissolving with the aid of heat 1 Gm. of purified benzidine in 20 c.c. of glacial acetic acid. Add 30 c.c. of distilled water and 50 c.c. of 95 per cent ethyl alcohol.

Test by placing 2 c.c. of the reagent in a test tube, add 1 c.c. of distilled water and 1 c.c. of 0.6 per cent hydrogen peroxide, and mix. No color, or only a very slight yellow color, should develop in two hours. If the mixture is then diluted to 25 c.c. with 20 per cent acetic acid and viewed through a thickness of about 1 cm., it should be practically indistinguishable from distilled water.

was plotted, as was done in the original paper.<sup>2</sup> The operations necessary for obtaining the data for the curve are as follows:

1. Weigh thirty-two 1 Gm. portions of blood-free human feces on an analytical balance and place each portion in a clean, dry, 50 c.c. centrifuge tube.

2. Make a 1:10 dilution of a blood sample of known hemoglobin content with distilled water and mix thoroughly.

3. Number each centrifuge tube and carefully add the required amounts of diluted blood to each of the 1 Gm. portions of feces, setting up the tubes in triplicate for each point on the curve to be determined. Mix the blood solution thoroughly with the feces, using individual glass stirring rods, and leave the rods in the tubes. Use the remaining two samples as duplicate blanks with blood omitted.

4. Add 10 c.c. of distilled water to the blanks and proportionately less to the remaining tubes, so that the total volume of fluid added will be 10 c.c. Mix thoroughly.

5. Add 15 c.c. of glacial acetic acid to each tube and mix. Heat and remove each tube as soon as the contents begin to boil vigorously. Care must be taken to prevent the contents from boiling over. (The heating was facilitated by putting tubes in wire baskets and placing them on an electric hot plate.)

6. Push particles adhering to the walls of the tubes back into the solution, remove the stirring rods, and arrange them according to number on the edge of a table with the acid-covered ends projecting so that none of the material on the rods may be lost. Centrifuge the tubes at approximately 2,700 r.p.m. for five minutes.

7. Decant the supernatant fluid into properly numbered 50 c.c. volumetric flasks. After transferring the contents of each tube, wash off the decanting rod with a small amount of glacial acetic acid.

8. Make three similar extractions, each with 10 c.c. of glacial acetic acid, and bring the contents of the 50 c.c. volumetric flasks to volume with glacial acetic acid and mix thoroughly.

9. Put 1 c.c. aliquots from the 50 c.c. volumetric flasks into 10 c.c. volumetric flasks, and bring the contents of the 10 c.c. volumetric flasks to volume with glacial acetic acid and mix.

10. Put 2 c.c. of the purified benzidine reagent into the bottom of each colorimeter tube, and add 1 c.c. aliquots from the 10 c.c. volumetric flasks and mix.

11. Add 1 c.c. of a 0.6 per cent hydrogen peroxide solution (3 per cent commercial solution freshly diluted with 4 parts of distilled water) and mix. Close with paraffined corks and allow the tubes to stand for two hours.

12. Dilute with 20 c.c. of a 20 per cent solution of glacial acetic acid in distilled water, insert corks, and invert the tubes several times to mix the contents. Allow to stand for at least eight minutes.

13. Adjust the colorimeter so that the blank reads 100 on the galvanometer scale when a No. 660 red filter is used. With this adjustment make the colorimetric readings on solutions derived from the feces-blood mixtures, and plot the average readings of the triplicates against the volumes of blood employed.

After we had been on a meat-free diet for at least three days, the volumes of blood (indicated in Table I) from the same calf that furnished the specimens for making the reference curve were diluted with 50 c.c. of distilled water and ingested. Each stool was then collected, weighed, thoroughly mixed by means of a spatula, and analyzed quantitatively for blood pigment according to the procedure outlined. Additional blood, however, was not added to the feces, 10 c.c. of distilled water were added to the blank and also to the feces containing blood, and one blank and triplicate samples of the unknown were sufficient for the analysis. The galvanometer readings obtained from the fecal extract containing the unknown quantity of blood were then translated into cubic centimeters of blood by means of the reference curve.

## RESULTS

The results of the analysis of the fecal specimens passed subsequent to the ingestion of calf's blood containing 9 Gm. of hemoglobin per 100 c.c. are shown in Table I.

TABLE I

QUANTITY OF INGESTED CALF BLOOD CONTAINING 9 GM. OF HEMOGLOBIN PER 100 C.C.  
RECOVERED FROM HUMAN FECES

| EXPERIMENT NO. | PERSON INGESTING BLOOD; DATE | VOLUME INGESTED | DAY | TIME INGESTED | TIME STOOL PASSED | HOURS AFTER INGESTION | WEIGHT OF STOOL | AVERAGE GALVA-<br>NOMETER READING | BLOOD INDICATED<br>PER GM. OF FECES | BLOOD RECOVERED | TOTAL BLOOD<br>RECOVERED |             |
|----------------|------------------------------|-----------------|-----|---------------|-------------------|-----------------------|-----------------|-----------------------------------|-------------------------------------|-----------------|--------------------------|-------------|
|                |                              | c.c.            |     | hour          | hour              | number                | Gm.             |                                   | c.c.                                | c.c.            | c.c.                     | per<br>cent |
| 1              | J. O. G.<br>May 23,<br>1941  | 61              | 1   | 3:30 P.M.     | 5:00 P.M.         | 1.5                   | 63              | 93                                | 0.05                                | 3               |                          |             |
|                |                              |                 | 2   | -----         | 9:30 A.M.         | 18.0                  | 971             | 93                                | 0.05                                | 49              |                          |             |
|                |                              |                 | 3   | -----         | 2:00 P.M.         | 46.5                  | 244             | 100                               | 0.00                                | 0               | 52                       | 85          |
| 2              | J. O. G.<br>June 3,<br>1941  | 65              | 1   | 2:30 P.M.     | -----             | -----                 | -----           | -----                             | -----                               | -----           | -----                    |             |
|                |                              |                 | 2   | -----         | 9:00 A.M.         | 18.5                  | 154             | 70                                | 0.17                                | 26              |                          |             |
|                |                              |                 | 3   | -----         | 1:30 P.M.         | 47.0                  | 20              | 76                                | 0.13                                | 3               |                          |             |
|                |                              |                 | 4   | -----         | 9:00 A.M.         | 66.5                  | 53              | 89                                | 0.07                                | 4               |                          |             |
|                |                              |                 | 5   | -----         | 9:00 A.M.         | 90.5                  | 100             | 100                               | 0.00                                | 0               | 33                       | 51          |
| 3              | J. S. A.<br>June 3,<br>1941  | 65              | 1   | 2:30 P.M.     | -----             | -----                 | -----           | -----                             | -----                               | -----           | -----                    |             |
|                |                              |                 | 2   | -----         | 8:30 A.M.         | 18.0                  | 136             | 89                                | 0.07                                | 10              |                          |             |
|                |                              |                 | 3   | -----         | 9:30 A.M.         | 43.0                  | 159             | 80                                | 0.10                                | 16              |                          |             |
|                |                              |                 | 4   | -----         | 9:00 A.M.         | 66.5                  | 95              | 100                               | 0.00                                | 0               | 26                       | 40          |
| 4              | J. S. A.<br>May 23,<br>1941  | 61              | 1   | 3:30 P.M.     | -----             | -----                 | -----           | -----                             | -----                               | -----           | -----                    |             |
|                |                              |                 | 2   | -----         | 9:00 A.M.         | 17.5                  | 65              | 93                                | 0.05                                | 3               |                          |             |
|                |                              |                 | 3   | -----         | 9:00 A.M.         | 41.5                  | 52              | 72                                | 0.16                                | 8               |                          |             |
|                |                              |                 | 4   | -----         | 9:00 A.M.         | 65.5                  | 93              | 83                                | 0.10                                | 9               |                          |             |
|                |                              |                 | 5   | -----         | No specimen       | -----                 | -----           | -----                             | -----                               | -----           | -----                    |             |
|                |                              |                 | 6   | -----         | 8:30 A.M.         | 110.5                 | 95              | 100                               | 0.00                                | 0               | 20                       | 33          |

The data in Table I show that 33 to 85 per cent of the ingested blood was recovered from the feces. All the specimens containing any appreciable quantity of blood were noticeably darker in color than the blood-free feces, but none of them in our opinion could be called "tarry." In Experiment 1 the blood either acted as a purgative or was accompanied by some substance which had a similar action. The rapid removal of the blood in this instance resulted in the recovery of 85 per cent within eighteen hours after ingestion. The re-

maining experiments show a progressively smaller percentage of recovery, and in general, indicate a negative correlation between the length of time the blood was retained in the digestive tract and the quantity recovered from the feces. Bramkamp<sup>5</sup> reported that pancreatic digestion *in vitro* reduced the strength of the color reaction between hemoglobin and benzidine by 50 per cent. This prolonged action of digestive juices plus the increased absorption of the blood from the intestine in those instances where the blood was retained in the digestive system for several days would explain the decrease in the apparent quantity of hemoglobin recovered.

A practical test of the method was made on the feces of a patient in the hospital of the School of Tropical Medicine who was suffering from profuse hemorrhage resulting from a condition diagnosed as ulcerative colitis. The results of these tests which were made during the time the patient was hospitalized and placed on a bland ulcer diet with rest in bed are shown in Table II. Because of the large amount of blood pigment in the first two fecal specimens obtained from this patient, four additional extractions with glacial acetic acid were necessary before all the hemoglobin was removed. In this instance the acid extracts were decanted into a 100 c.c. volumetric flask. The blood pigment was so highly concentrated in the extract that it was necessary to use a fraction of a cubic centimeter of the diluted extract in order to obtain a color intensity that could be measured in the colorimeter; hence the multiplying factors shown in column 5.

TABLE II  
QUANTITY OF BLOOD RECOVERED FROM THE FECES OF A PATIENT SUFFERING  
FROM ULCERATIVE COLITIS

| DATE     | WEIGHT<br>OF<br>STOOL | AVERAGE<br>GALVAN-<br>OMETER<br>READINGS | BLOOD<br>INDI-<br>CATED | MULTI-<br>PLYING<br>FACTOR | BLOOD*<br>INDICATED<br>IN ONE<br>GM. OF<br>FECES | TOTAL<br>BLOOD*<br>RECOVERED |
|----------|-----------------------|--|-------------------------|----------------------------|--|------------------------------|
|          | Gm.                   |  | c.c.                    |                            | c.c.   | c.c.                         |
| 10/10/40 | 150                   | 53                                       | 0.275                   | 16                         | 4.40   | 660                          |
| 10/11/40 | No specimen           |  |                         |                            |  |                              |
| 10/12/40 | 107                   | 93                                       | 0.050                   | 8                          | 0.40   | 43                           |
| 10/13/40 | 135                   | 89                                       | 0.070                   | 0                          | 0.07   | 9                            |
| 10/14/40 | 90                    | 108                                      | 0.000                   | 0                          | 0.00   | 0                            |

\*Volume of blood computed on the basis of 9 Gm. of hemoglobin per 100 c.c.

The data in Table II show clearly the severity of the hemorrhage at the beginning of treatment and the gradual cessation of the bleeding under proper care.

#### DISCUSSION

At first glance the time and technical difficulties involved might make the method outlined appear to be impractical. However, we found that once the reference curve was made, the routine was so well known that tests on specimens containing unknown quantities of blood were easily and quickly carried out. One and one-half hours would probably be a conservative estimate of the time consumed in running a test consisting of one blank and triplicate unknowns. This period does not include the time required for color development.

Five points were emphasized as being important in the successful carrying out of the procedure for the quantitative determination of blood in the feces of sheep. These points are:

1. Use the same lot of purified benzidine reagent for plotting the reference curve and for making the determinations on the unknown in any one experimental series.
2. Always make determinations on fresh fecal specimens.
3. Use meticulous care in weighing, pipetting, and diluting operations.
4. Use fresh glacial acetic acid fecal extracts in making colorimetric comparisons.
5. Do not use blanks for reference points for any other similar sample containing blood unless the two tubes have been diluted at the same time.

In the application of the method to human feces the observance of these measures is just as important as it was in the previous work. The reader is referred to the original paper for further discussion.

#### SUMMARY AND CONCLUSIONS

A modification of a method for the quantitative determination of blood in the feces of sheep is described for use with human feces. By its use 33 to 85 per cent of ingested blood was recovered from the feces. A negative correlation was observed between the percentage recovered and the length of time the blood remained in the digestive tract. This decrease in the amount of blood recovered was considered to be due to the action of digestive juices and to absorption within the digestive tract.

The method was tested on the feces of a patient bleeding profusely from a condition diagnosed as ulcerative colitis. During the period the patient was under treatment, the tests demonstrated the severity of the hemorrhage at the beginning of treatment and the gradual cessation of bleeding during the days that followed until no more fecal blood was demonstrable.

The results of the tests indicate that the method described can be used for the purpose of estimating the amount of blood passed in the feces of human patients suffering from gastrointestinal hemorrhage.

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# THE ADAPTATION OF MARSHALL'S MICRO SULFONAMIDE METHOD TO THE PHOTOELECTRIC COLORIMETER\*

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THE increasing importance of the sulfonamides in the treatment of various bacterial infections has resulted in the development and extensive use of several methods for determining these substances in the blood, urine, and body tissues. Of these methods those of Marshall,<sup>6</sup> and Bratton and Marshall,<sup>2</sup> are perhaps the most extensively used in this country. In this report Marshall's improved method<sup>6</sup> has been adapted for use in the determination of sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, and sulfaguanidine† with the Evelyn photoelectric colorimeter<sup>4</sup> which is a stabilized direct reading single-photocell type instrument.

The introduction by Bratton and Marshall of N-(1-naphthyl) ethylenediamine dihydrochloride as the coupling reagent to replace the dimethyl alpha-naphthylamine, which had been previously employed, has eliminated some of the objections to the Marshall method advanced by Andrews and Straus.<sup>1</sup> Sunderman and Pepper<sup>7</sup> reported a diminution in recovery of sulfathiazole from whole blood when using the coupling agent (dimethyl-alpha-naphthylamine) of Marshall's original procedure. It is now easily possible to complete a determination by Marshall's method in approximately fifteen minutes, and the color produced remains stable for a period of two or more hours. The method recently proposed by Churg and Lehr,<sup>3</sup> while equally rapid for the free sulfonamide, is somewhat slower for the total determination than the procedure presented here.

The method to be described has proved rapid, simple, and accurate, as evidenced by high percentage recoveries of the various sulfonamides which have been added to whole blood (dog and human), and may be used with either 0.2 or 0.1 c.c. of blood. Certain previous objections or uncertainties of the method, such as the length of time required for analysis, instability of color, or incomplete recoveries, are eliminated by the use of Marshall's more recent coupling agent N-(1-naphthyl) ethylenediamine dihydrochloride and by the use of the photoelectric colorimeter.

*Reagents.*—1. A solution of trichloroacetic acid containing 15 Gm. dissolved in water and diluted to 100 c.c.

2. A 0.1 per cent aqueous solution of sodium nitrite—this reagent should preferably be made up at weekly intervals.

\*From the Aero Medical Unit, Army Air Forces, Materiel Center, Wright Field, Dayton.

†Supplies of these sulfonamides were obtained from Merck & Co., Inc., and the Lederle Laboratories, Inc.

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3. An aqueous solution of N-(1-naphthyl) ethylenediamine dihydrochloride containing 100 mg. per 100 c.c. This solution should be kept in a dark-colored bottle.

4. 4 N hydrochloric acid.

5. A solution containing 0.5 Gm. of ammonium sulfamate per 100 c.c. This solution should be freshly prepared each week and discarded at the first sign of contamination with molds.

6. Stock solutions of the various sulfonamides in water containing 200 mg. per liter. From this solution a series of dilutions were prepared for use in determining the respective calibration curves.

#### PROCEDURE

*Blood for 0.2 c.c. Samples.*—Two-tenths cubic centimeter of blood (oxalated or heparinized) is delivered into a colorimeter tube\* containing 7.8 c.c. of distilled water. Proteins are precipitated by adding 2.0 c.c. of trichloroacetic acid, and removed by filtering. (Whatman No. 12 has proved suitable.)

*Blood for 0.1 c.c. Samples.*—One-tenth cubic centimeter of blood is added to 3.9 c.c. of distilled water in a graduated centrifuge tube and 1.0 c.c. of trichloroacetic acid is added for protein precipitation. Centrifuge for four to five minutes. Two cubic centimeter samples of supernatant fluid are then treated as outlined for the free and total sulfonamide determination.

*Free Sulfonamide.*—Two cubic centimeter samples of the blood filtrate are delivered into a colorimeter tube. One-half cubic centimeter of sodium nitrite is added. After two minutes 0.5 c.c. of the ammonium sulfamate is added. After another two minutes, 0.5 c.c. of N-(1-naphthyl) ethylenediamine dihydrochloride is added. The solution is then diluted to 10 c.c. by adding 6.5 c.c. of distilled water, and the color density is determined in the photoelectric colorimeter with a green filter (Corning) No. 540.

*Total Sulfonamide.*—After the sample for determination of the free sulfonamide is removed, the remainder of the blood filtrate is placed in a graduated centrifuge tube, the volume is observed and 0.4 c.c. of 4N HCl is added. It is heated in boiling water for twenty minutes, cooled, and adjusted to original volume with distilled water. This dilution precaution must be observed to avoid considerable error. Subsequent procedure is identical to that outlined above for free determination.

*Calibration Curves.*—Dilutions of a stock solution (200 mg. per liter) were prepared in concentrations ranging from 0.5 to 15.0 mg. per 100 c.c. and containing 18 c.c. of trichloroacetic acid per 100 c.c. A 0.2 c.c. sample of these respective stock dilutions was added to 8.1 c.c. of distilled water to which 0.2 c.c. of trichloroacetic acid has been added. One-half cubic centimeter of each reagent was then added at two-minute intervals. The calibration curves of the respective sulfonamides were then determined from the colorimeter readings of samples prepared in duplicate as above.

\*These tubes are of especially selected glass and are furnished by the Rubicon Company, Philadelphia, Pa. from whom the Evelyn photoelectric colorimeter and filters may also be obtained.

*Reagent Blank.*—A stock blank is prepared by adding 2.0 c.c. of trichloroacetic acid to 8.0 c.c. of distilled water. A 2.0 c.c. sample is then delivered into a colorimeter tube, and 0.5 c.c. of each of the reagents is added at two-minute intervals. Six and one-half cubic centimeters of distilled water are added, the tube is placed in a colorimeter and the galvanometer is adjusted to 100, the reagent blank thus serving as the 0.0 mg. per 100 c.c. standard.

## RESULTS

Calibration curves relating concentration (milligrams) to photoelectric density ( $2 - \log G$ ) of these sulfonamides were found to be linear from a concentration of 0.5 mg. per 100 c.c. to 10 mg. per 100 c.c. for all the sulfonamides tested. Concentrations in excess of 10.0 mg. per 100 c.c. deviate somewhat from a straight line, particularly in the case of sulfanilamide. The calibration factors ( $K$ ) derived from the respective curves were used in the calculation of the per cent recoveries of the various sulfonamides from whole blood. These factors were 50.6 for sulfanilamide, 33.5 for sulfapyridine, 36.0 for sulfathiazole, 36.9 for sulfadiazine, and 39.3 for sulfaguanidine. Standard deviations of these factors were 1.2, 1.2, 0.2, 0.2, and 0.2, respectively.

The percentage error for the 0.2 c.c. procedure in the recoveries from whole blood concentrations of 10 mg. per 100 c.c. varied from 0.2 to 2 per cent, and from 1 to 2 per cent in recoveries from concentrations of 1 mg. per 100 c.c. This probably represents the limit of accuracy for the 0.2 c.c. method as described here. The accuracy of the 0.1 c.c. procedure is somewhat less.

Results reported here were calculated by use of the formula:

$$\text{Mg. per 100 c.c.} = \frac{(2 - \log G)}{K} \times \frac{\text{dilution used}}{\text{c.c. of filtrate used}} \times 100$$

$G$  is the galvanometer reading and  $K$  the calibration factor for the respective sulfonamide. For a 1:50 blood dilution the use of 2 c.c. blood filtrates and the twenty-minute hydrolysis period have been found to give satisfactory results. Color developed in the blood blank is negligible, since in the 1:50 dilution of blood the correction due to this was found to be about 0.08 mg. per 100 c.c. The reagent blank shows no measurable color development subsequent to its exposure to ordinary indoor room lighting conditions over a period of three to four hours. If analyses continue beyond this period of time, a fresh blank should be prepared if precise results are desired. The color developed as a result of the coupling reaction is stable for a period of two or three hours, after which its density slowly alters, and readings made after longer periods of time may be considerably in error.

## DISCUSSION

The use of calibration curves for the respective sulfonamides seems desirable, since they are linear over the range of 0.5 to 10 mg. per 100 c.c. and quite reproducible. Their use avoids any uncertainty that arises from the employment of a standard whose color density, as mentioned above, is slowly altering. The green filter recommended (Corning No. 540) may be used for all the sulfonamides reported, since the wave length of the maximum absorption for

the respective compounds has been found by Feinstein and associates<sup>5</sup> to be practically identical. As pointed out by Bratton and Marshall<sup>2</sup> the method is extremely sensitive, and concentrations of 1  $\mu$ g can be easily detected.

Diazotization with the nitrite and destruction of the excess nitrite by the ammonium sulfamate are rapid reactions, both being complete within thirty to sixty seconds. Formation of the azo dye by the coupling reaction is slower and the two-minute interval recommended must be observed for reproducible results. Complete analyses for the free drug require twelve to fifteen minutes, while the total sulfonamide determination may be completed in thirty-five to forty minutes.

N-(1-naphthyl) ethylenediamine dihydrochloride as the coupling agent possesses several distinct advantages, some of which have previously been pointed out by Bratton and Marshall. One of these is its speed, the method reported here being equally as rapid as that suggested by Andrews and Straus,<sup>1</sup> or Churg and Lehr.<sup>3</sup> The procedure proposed by the latter workers requires also the undesirable use of an empirical factor to correct for a decrease in the color intensity due to the additions of hydrochloric acid in the total sulfonamide determination. No difficulty in securing satisfactory recoveries of sulfathiazole added to whole blood has been experienced. The necessity, therefore, for centrifuging the protein precipitate as Sunderman and Pepper<sup>7</sup> reported, has been removed.

It is important in all procedures to observe the time interval between the addition of the various reagents and also to agitate slightly the solution during the various steps. Unless these precautions are observed in the preparation of blanks or samples, turbidities may result, or improper unstable colors may develop.

#### SUMMARY

1. An adaptation of Bratton and Marshall's method for the determination of sulfonamides in 0.2 and 0.1 c.c. blood samples is described for use with the photoelectric colorimeter.

2. Calibration curves obtained with the Evelyn photoelectric colorimeter over a 0.5 to 15 mg. per 100 c.c. range of concentration has been determined for sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, and sulfaguanidine. The calibration factors (K) and standard deviations of these curves are presented.

3. The per cent error in the recoveries of these various sulfonamides as determined in concentrations of 1 and 10 mg. per 100 c.c. varied from 0.2 to 2.0 per cent.

4. The method as presented here is simple, sensitive, relatively rapid, and gives precise reproducible results over a range of concentrations varying from 0.5 to 15.0 mg. per 100 c.c.

I wish to express my appreciation to Dr. J. W. Heim for helpful criticism and suggestion.

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## A CLINICAL MICROMETHOD FOR THE DETERMINATION OF BLOOD UREA\*

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JOSEPH C. BOCK, CH.E., PH.D., MILWAUKEE, WIS.

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THE diagnostician realizes that functional disturbances may be the first indication of anatomic changes. A deviation from normal nonprotein nitrogen of the blood is often a sign of a metabolic disturbance, if not an indication of a change to a definite pathologic stage. The study of the total nonprotein nitrogen or of urea nitrogen in the blood, although of diagnostic value in a number of disturbances, is most important when confronted with the problems of renal dysfunction.

The determination of the total nonprotein nitrogen of the blood, while most frequently employed, has several disadvantages. The method requires a well-equipped laboratory and a technician with good training. The nonprotein nitrogen fraction of the blood contains about 25 per cent of undetermined or rest nitrogen, made up of nitrogenous substances of practically unknown metabolic significance, especially under pathologic conditions. The relative increase in urea nitrogen is greater than the nonprotein nitrogen rise when dealing with certain renal disturbances. It must be kept in mind that urea is largely a waste product, whereas the undetermined nitrogen is anabolic. We believe, therefore, that the variations in urea alone serve as a better diagnostic guide than the nonprotein nitrogen.

A recently published procedure<sup>1</sup> for the determination of urea nitrogen has been modified to give the physician a method which does not require a laboratory, and at the same time is accurate enough to satisfy his diagnostic needs.

The enzyme urease converts urea into ammonium carbonate. The addition of the alkali sodium carbonate liberates ammonia, which is absorbed in the present method through "passive distillation" in hydrochloric acid.

The apparatus consists of a cylindrical vessel and a small glass cup fused to a rod. The cup and rod are attached to a cork which is inserted into the cylinder (Fig. 1).

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The procedure is as follows: 0.1 ml. of blood from the ear lobe or finger tip is measured into the cup. One urease tablet, containing both urease and the necessary buffer salts, is crushed, and one-third to one-half of this powder is added to the blood contained in the cup. Blood and urease are mixed by gentle agitation, care being taken to avoid any liquid spilling over the rim of the cup. The stopper with the enp is inserted into the vessel and is allowed to stand for about one hour at room temperature. The enp assembly is then

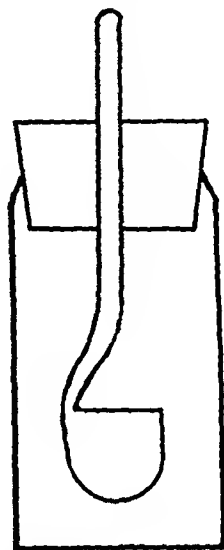


Fig. 1.

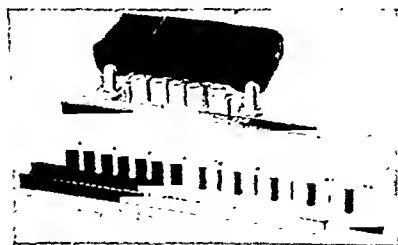


Fig. 2.

removed, and 1 ml. of approximately tenth-normal hydrochloric acid is carefully measured into the cylinder. Three drops of a concentrated solution of sodium carbonate are added to the blood in the cup, and the stopper is *quickly* inserted, to avoid any possible loss of ammonia. The vessel is gently agitated by a rotary motion, to mix the contents of the cup. The apparatus is allowed to stand (at room temperature) for about eight hours, or still better overnight. The stopper with the cup is then removed, 3 ml. of water are added to the fluid in the cylinder and the contents are mixed by rotation. One milliliter of Nessler reagent (Bock-Benedict modification<sup>1</sup>) is added, the solutions are mixed again and allowed to stand for eight to ten minutes. This is necessary for full development of the color. The liquid is transferred to a small tube, which in

turn is inserted into the holder of a Taylor\* slide comparator (Fig. 2). The movable slide of the instrument has a set of tubes containing permanent standards, the colored tubes being interspersed by tubes containing water. The slide is moved back and forth until the unknown is flanked by those two standard tubes which match the closest. The standards are permanent, consisting of a mixture of ferric and cobaltous chloride in acid solution and arranged to give a direct reading in milligrams per 100 c.c. of blood, ranging from 10 mg. to 100 mg. of nitrogen.

The color standards are made as follows:

$\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$  : 100 Gm. in 1,000 c.c., using 1 per cent (by weight) hydrochloric acid as solvent.

$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  : 100 Gm. in 1,000 c.c., using 1 per cent (by weight) hydrochloric acid as solvent.

| UREA NITROGEN<br>(MG. PER 100 C.C.) | $\text{FeCl}_2$<br>SOLUTION<br>(C.C.) | $\text{CoCl}_2$<br>SOLUTION<br>(C.C.) |  |
|-------------------------------------|---------------------------------------|---------------------------------------|--|
| 10                                  | 11.30                                 | 4.35                                  | Make total volume to<br>100 c.c., using 1 per<br>cent (by weight) hy-<br>drochloric acid solu-<br>tion |
| 20                                  | 20.45                                 | 9.45                                  |  |
| 30                                  | 29.50                                 | 13.70                                 |  |
| 40                                  | 37.05                                 | 15.55                                 |  |
| 50                                  | 50.30                                 | 21.55                                 |  |
| 60                                  | 52.70                                 | 26.35                                 |  |
| 70                                  | 54.35                                 | 31.15                                 |  |
| 80                                  | 56.00                                 | 36.00                                 |  |
| 100                                 | 54.55                                 | 45.50                                 |  |

#### REFERENCE

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\*The complete equipment may be obtained from W. A. Taylor and Company, 7300 York Road, Baltimore, Md.

# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**ALCOHOL**, The Perchlorate Method for Determining Concentration of, in Expired Air, Jetter, W. W., and Forrester, G. C. *Arch. Path.* 32: 829, 1941.

A critical evaluation of the perchlorate method for the determination of breath alcohol has been made, and the following data have been presented: When the test is properly made, there is no loss of alcohol or of carbon dioxide before the breath reaches the absorption tubes; reducing substances in the breath other than alcohol are negligible in their effect; the presence of acidosis or alkalosis in the test subject materially affects results, but these conditions may be controlled or eliminated by proper precautionary measures.

In a series of 79 cases in which breath and direct blood analyses were made, the deviation did not exceed 16 per cent in any instance.

In view of the simplicity of operation and the ease with which the instrument can be transported, this method is well adapted for use by law enforcement agencies.

**AMYLOIDOSIS**, Standardization of the Congo Red Test for, Taran, A., and Eckstein, A. *Am. J. M. Sc.* 203: 246, 1942.

A standardized method for the Congo red test for amyloidosis is presented.

The dye is administered according to body weight. It was found that 1 c.c. of a 1 per cent aqueous solution of the dye per 10 pounds of body weight yielded more accurate tests than the fixed amount customarily used.

Tests were performed on amyloid and nonamyloid patients, using both the usual technique of injecting 10 c.c. of dye regardless of body weight and the standardized method.

In one nonamyloid group patients were selected whose body weights were nearly the same (range between 95 and 105 pounds) and in the other those whose body weights varied considerably (range between 50 and 175 pounds). The former group showed consistently reliable four-minute specimens, while the latter gave four-minute specimens which differed as much as 75 per cent. In some cases the per cent absorption could not be computed because of insufficient dye in the four-minute specimens.

In the amyloid group 90 to 100 per cent absorption was a consistent finding. This is in so close correlation with previous work that such results must be obtained to indicate amyloidosis. In all but 11 cases the four-minute specimens contained sufficient dye to be considered as appropriate standards.

These 11 cases were investigated further along with 24 of the other amyloid cases, and it was found expedient to draw a two-minute specimen in addition to the four-minute and one-hour specimens. A group of 60 nonamyloid cases was chosen as controls. In all of the amyloid cases, the two-minute specimens showed good color intensity and the differences in color between them and the four-minute specimens were marked (as high as 75 per cent). There were only slight differences in color intensity between the two-minute and four-minute specimens in the nonamyloid group, the highest per cent variation being 15.

The two-minute specimen was adopted as the colorimetric standard in place of the four-minute specimen.

Since the inception of this study 21 of the 42 amyloid patients have come to autopsy and each revealed amyloidosis of greater or lesser degree. Twenty of the nonamyloid patients have come to autopsy and only one case revealed a moderate degree of amyloidosis.

The standardized method has improved the accuracy and reliability of the test as a diagnostic aid.

The authors' method follows:

Inject 1 c.c. of a 1 per cent aqueous solution of Congo red per 10 pounds of body weight intravenously. At the end of exactly four minutes, and then one hour, after the injection, obtain blood specimens of 10 c.c. each and place them into clean, dry test tubes. Allow the bloods to clot and retract and then centrifuge them at a moderate speed for ten minutes. Aspirate off the clear sera and place them into graduated centrifuge tubes. Add acetone equal to the volume of serum in each tube and shake them well. Centrifuge tubes for ten minutes at a moderate speed. Pour off the supernatant fluids and place them into micro cups of a colorimeter to be compared. Set the four-minute specimen at 20 mm. and read the one-hour specimen.

Calculation:

$$100 - \frac{\text{Reading of four-minute specimen}}{\text{Reading of one-hour specimen}} \times 100 = \text{Per cent absorption.}$$

For example: The four-minute specimen is set at 20 mm. in the colorimeter. The one-hour specimen reads 25. Therefore, the per cent absorption of the dye is 20.

**COAGULATION, Blood, Simple Method of Timing, Knauer, C. H.** J. Med. Soc. New Jersey 39: 75, 1942.

An ordinary hypodermic syringe with a slightly larger-sized needle than ordinary is used. If necessary, the small diameter needle will serve. Blood is withdrawn from the median basilic or some other prominent vein. A few bubbles of air are drawn into syringe, and it is allowed to rest on its side for three minutes, after which time it is tilted slowly from end to end so as to permit the bubbles with their interspaces of blood to travel slowly from one end of the barrel to the other at one-quarter minute intervals. It is soon found that the rapidity with which these bubbles travel the length of the syringe becomes diminished with the formation of the clot and that one-half to three-quarters minute transpires between the onset of the clot formation and the actual completion of the process which is noted by the almost immobility of the bubbles.

**RHEUMATIC FEVER, Formol-Gel Test in, Butterworth, J. S., and Poindexter, C. A.** Am. J. M. Sc. 203: 278, 1942.

The formol-gel test is essentially a test for hyperglobulinemia and has no correlation with the erythrocyte sedimentation rate.

It is of little or no value in the diagnosis of acute rheumatic fever with or without carditis.

**HYPOPROTHROMBINEMIA in Pernicious Anemia, Warner, E. D., and Owen, C. A.** Am. J. M. Sc. 203: 187, 1942.

Patients with Addisonian pernicious anemia in relapse usually show considerable decrease in plasma prothrombin. In the majority of such cases the prothrombin level is found to be between 40 per cent and 65 per cent of the normal. The hypoprothrombinemia is not rectified by large doses of vitamin K. When specific liver therapy is instituted, the plasma prothrombin level promptly shows a marked rise.

**PROTHROMBIN DEFICIENCY, Effect of Vitamin K Therapy on the Plasma Prothrombin Level of Patients With, Rhoads, J. E., and Norris, R. F.** Bull. Ayer Clin. Lab., Pennsylvania Hosp. 3: 359, 1941.

The administration of an excess of 2 methyl-1,4-naphthoquinone to surgical patients without hypoprothrombinemia resulted in a slight shortening of the prothrombin time, as determined by the Quick method and a modification thereof.



This difference is not regarded as significant in the number of cases studied, in view of the variations observed among the controls; it is less than the difference between normal controls and the plasma of parturient mothers, observed by Norris and Rush, and much less than was observed in some of their patients.

It is also of interest that six days of therapy yielded no better result than did two days of therapy.

The results indicate, therefore, that the supply of vitamin K is not an important limiting factor in determining the prothrombin level under normal conditions.

There is nothing in the results of the study to suggest that the administration of vitamin K would be valuable in the prevention of hemorrhage in patients who do not have hypoprothrombinemia, nor that it would be dangerous to give the vitamin in considerable excess to patients whose prothrombin levels were already normal.

The method of study is not sufficiently accurate to establish satisfactorily or to exclude a mass action effect of vitamin K on the production of prothrombin.

**PROTHROMBIN, Comparison of Bilirubin Values and, in the Umbilical Artery and Vein at Delivery, Norris, R. F., and Bennett, M. C. Bull. Ayer Clin. Lab., Pennsylvania Hosp. 3: 353, 1941.**

The average bilirubin content of fetal blood exceeds that of maternal blood at delivery. In individual infants there is a marked variation in the degree of hyperbilirubinemia, which is independent of the amount of bilirubinemia in their respective mothers.

There is no evidence that bilirubin diffuses in either direction through the placenta.

No relationship is demonstrated between the amount of serum bilirubin and the plasma prothrombin in individual infants.

**SHOCK, The Vascular and Cellular Dynamics of, Moon, V. H. Am. J. M. Sc. 203: 1, 1942.**

An analysis of the sources for disagreement concerning the dynamics of shock indicates that four major causes have operated to confuse interpretations of its phenomena and of their interrelationships.

The first of these was deficient knowledge of capillary reactions and of circulatory disturbances originating in them. A comprehension of these has contributed immeasurably to an understanding of shock resulting from trauma, burns, and other types of tissue damage. It clarifies also the disturbances of fluid balance and of chemical concentrations which are associated regularly with this condition.

Failure to distinguish between shock and hemorrhage has caused much confusion. Hemorrhage, when present, is a potent contributory factor, but the effects of uncomplicated hemorrhages present numerous features opposite in character from those which constitute the syndrome of shock.

Variations in blood pressure used as a criterion, and failure to consider the depressor effects of both anesthesia and losses of blood, have led to undependable conclusions concerning the effects of absorption. Varied forms of experimentation have shown that products derived from normal tissues, independent of narcosis and hemorrhage, will produce the syndrome of shock accompanied by its characteristic physiologic and morphologic features.

Hemoconcentration during life, and the presence of engorged capillaries, stasis, petechiae, and edema after death, indicate endothelial damage as a major factor. The occurrence of these conditions in extensive visceral areas remote from the injury indicates systemic, not local, endothelial damage. Shock, like other conditions of disease, is accompanied by a pattern of morphologic changes which are related etiologically to its mechanism of origin and which corroborate the interpretation of shock as due to endothelial damage.

The major causes for disagreement concerning shock have been discussed. In view of the character and magnitude of these, it is not strange that final clarification of the problem has been delayed.

## REVIEWS

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Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

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### Lymphatics, Lymph, and Lymphoid Tissue\*

A COMPREHENSIVE authoritative volume, this book covers the origin, nature, and function of the elements indicated in the title.

As the writers bring out, study of the physiology of lymph carries us nearer to a study of the life processes of the tissue cells. Lymph circulates from the blood vessels into the tissue spaces surrounding the cells and out again through the lymphatics into the blood vessels. It is this fluid that is of utmost importance in the maintenance of vital cellular equilibrium. Considerable space is necessarily given to the phenomena of capillary permeability, and there is an associated discussion of water balance and electrolyte metabolism. Clinical implications are adequately discussed.

This volume should be of interest to physiologists and, on the clinical side, especially to surgeons, internists, allergists, and dermatologists.

### Photomicrography†

A HANDBOOK which adequately covers the entire field of photomicrography, the first portion of *Photomicrography* deals with the physics of photography, especially as applied to the use of the microscope with photographic cameras. The major portion of the book deals with equipment and its use. This extends from simple homemade equipment, through the use of candid camera attachments, such as the Leica and Contax, up to complicated apparatus of highest precision, including the electron microscope. Fifty pages are given to darkroom technique, developing and printing, and there is an abundance of formulas for developing solutions. In this way the book is complete, requiring little or no reference to other volumes. It is abundantly and very well illustrated.

### Biological Symposia‡

THIS volume is devoted entirely to the structure and experimental physiology of muscle. It contains sixteen essays by various authors. One is likely to find the answer to whatever one would wish to know concerning muscle physiology, somewhere in this volume. Unfortunately there is no index. Also, the binding does not indicate the subject matter. Although the book is devoted to one subject, muscle, one must look inside to discover this.

Subjects discussed include muscle excitability, action, potentials and conduction, activity around the neuromuscular junction, regulation of energy exchange, changes during muscle contraction, the significance of oxidation, efferent innervation, electrolyte equilibrium in muscle, and electric potential changes accompanying neuromuscular transmission. Both smooth and striated muscle are covered.

\*Lymphatics, Lymph, and Lymphoid Tissue. Their Physiological and Clinical Significance (Harvard University Monograph in Medicine and Public Health, No. 2). By Cecil Kent Drinker, M.D., D.Sc. Professor of Physiology, School of Public Health, Harvard University; and Joseph Mendel Yoffey, M.Sc., M.D., F.R.C.S. (Eng.), Senior Lecturer in Anatomy, University College of South Wales and Monmouthshire, Cardiff, Wales. Cloth, 406 pages, \$4.00. Harvard University Press, Cambridge, Mass., 1941.

†Photomicrography. By R. M. Allen. Cloth, 365 pages, \$5.50. D. Van Nostrand Company, New York, 1941.

‡Biological Symposia—A Series of Volumes Devoted to Current Symposia in the Field of Biology. Edited by Jaques Cattell, Editor of The American Naturalist and American Men of Science. Volume III. Muscle. Cloth, 370 pages. The Jaques Cattell Press, Lancaster, Pa., 1941.

## A Manual of Neurohistologic Technique\*

THIS small volume presents methods of preparation and staining of nerve tissue for microscopic study. In it the author has collected the best techniques from scattered places in the literature and gives his own critical experiences with them. This should make a handy reference volume for pathologists and neuroanatomists.

## An Introduction to Dermatology†

PRIMARILY a condensation of the larger book *Diseases of the Skin* by the same authors, the fourth edition of the book is based on the tenth edition of the larger volume. Even the introduction is large enough, covering 900 pages. It is abundantly and well illustrated, and constitutes an adequate dermatologic reference book for all who are not primarily dermatologists.

## Chemical Solutions‡

IN THIS book the author has collected in one place the methods for the preparation of the solutions most commonly used in the average chemical laboratory, such as organic, biochemistry, food chemistry, metallurgy and metallography, bacteriology, gas and fuel analysis, general testing, and water analysis. The solutions are listed alphabetically according to their most common name; when a reagent is known by several names, these names are also included in their proper place in the alphabetical tabulation with proper cross references. Additional valuable information is also given, such as, (a) the uses of each solution; (b) the procedure for use where this is practicable; (c) interfering substances; (d) sensitivity of the test reagent; and (e) general keeping qualities. One or more reference is included for each solution, wherever advisable. A valuable feature is an index in which the various reagents are classified according to their use. All descriptions are concise and to the point, and unnecessary detail is eliminated. It is the opinion of the reviewers that this book should prove a most valuable adjunct to any chemical library.

## Chromatographic Adsorption Analysis§

A GOOD technique sometimes renders more service to science than the elaboration of highly theoretical speculations." With this introduction from the writings of Claude Bernard, Dr. Strain in this book, Volume II of the series of monographs on Chemical Analysis by the same publishers, has compiled in a single volume practical information on chromatography from widely scattered sources. The remarkable influence of this technique on the progress of many chemical investigations of varied nature, particularly in the field of biological compounds, makes such a book highly desirable. The technique of separation of many types of compounds, organic and inorganic, colorless as well as colored, by adsorption on Tswett columns is described. Many diagrams of apparatus are presented. Various absorbents, their applications, limitations, availability, and choice for certain types of compounds, as well as various methods for filling the columns, are discussed. Selection of solvents and eluents and the many factors influencing their usefulness are presented in detail. The usefulness of the volume is greatly enhanced by the inclusion of an extensive bibliography, with titles from which one can readily find literature pertaining to particular problems, as well as a very good author and subject index which indexes bibliography as well as text. It is a useful

\*A Manual of Neurohistologic Technique. By Oscar A. Turner, M.D., New Haven, Conn. Cloth, 73 pages. The C. V. Mosby Company, St. Louis, Mo., 1940.

†An Introduction to Dermatology. By Richard L. Sutton, M.D., Sc.D., LL.D., F.R.S. (Edin.), Emeritus Professor of Dermatology, University of Kansas School of Medicine; and Richard L. Sutton, Jr., A.M., M.D., LL.R.C.P. (Edin.), Assistant Professor of Dermatology, University of Kansas School of Medicine. Cloth, ed. 4, 904 pages, with 723 illustrations. The C. V. Mosby Company, St. Louis, Mo., 1941.

‡Chemical Solutions. By Frank Veleher, Assistant Professor of Chemistry. 404 pages, \$1.75, D. Van Nostrand Company, Inc., New York, N. Y., 1942.

§Chromatographic Adsorption Analysis. By Harold H. Strain, Ph.D., Carnegie Institution of Washington, Stanford University, Calif. Cloth, 222 pages, 37 illustrations, colored frontispiece. \$3.75. Interscience Publishers, Inc., New York, N. Y., 1942.

practical book for the chemical investigator, and its readable style also makes it valuable for others who would like to know more about the technique involved in chromatographic adsorption analysis.

### **Analytical Chemistry of Industrial Poisons, Hazards and Solvents\***

**T**HIS is an exhaustive text dealing with the detection of substances important from the standpoint of industrial health. Thorough discussion is made of air sampling, measurement of gas volume and quantity, absorbers and absorbents, the chemical and microscopic estimation of dust. Then follows specific and detailed description of the detection of silica, the dangerous metals, poisonous compounds of sulfur, and the halogens. The gases, carbon monoxide, carbon dioxide, hydrocyanic, and cyanogen, are subsequently taken up and then a consideration of the various solvents, including the alcohols and glycols. The author closes his text on the very timely topic of the chemical warfare agents. Brief mention is made in each instance on the toxicity and physiologic activity of the materials under consideration. This is an outstanding text which will prove most beneficial to all interested in industrial health. It is most heartily recommended.

H. B. HAAG.

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\*The Analytical Chemistry of Industrial Poisons, Hazards and Solvents. By M. B. Jacobs. Food, Drug and Insect Administration, United States Department of Agriculture, 1927; Chemist, Department of Health New York City, 1928; formerly Lt. U. S. Chemical Warfare Service Reserve. 110 illustrations, 661 pages. Interscience Publishers, Inc., New York, 1941.

# The Journal of Laboratory and Clinical Medicine

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## CLINICAL AND EXPERIMENTAL

### HEMATOLOGIC VALUES FOR NORMAL HEALTHY MEN 16 TO 25 YEARS OF AGE\*

CHRISTOPHER J. HAMRE, PH.D., AND MAN HING AU, M.S.  
HONOLULU, T. H.

NUMEROUS investigations have been made in this country and in other countries during the past few years in an effort to determine the reliability of long-accepted blood values and to establish standard normal blood values for particular geographical areas. The greater number of studies have been concerned with the normal or mean values for the quantity of hemoglobin and the number of red blood cells for healthy persons. Only a relatively small number of studies have been made on the normal values for other blood elements. The present report is concerned with the results of complete hematologic examinations of healthy men. Reports in the literature of similar complete hematologic studies for men have not come to our attention.

Reviews, including normal values for all blood elements for adult men, are lacking, though reviews of the studies of normal values of some individual blood elements are found in the literature. Myers and Eddy,<sup>1</sup> and Nelson and Stoker,<sup>2</sup> have reviewed the literature on normal values for hemoglobin of men, while the latter, Andresen and Mugrage,<sup>3</sup> and Mugrage and Andresen<sup>4</sup> have reviewed the literature on normal numbers of red blood cells. The last-named authors have also presented a consideration of the values for the various corpuscular constants. Garrey and Bryan,<sup>5</sup> Osgood,<sup>6</sup> and Osgood and co-workers<sup>7</sup> have reviewed the literature on leucocyte values, while Olef<sup>8</sup> has reviewed

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TABLE I  
MEAN VALUES FOR HEMOGLOBIN, RED BLOOD CELLS, AND CORPUSCULAR CONSTANTS REPORTED FOR MEN

| OBSERVER                                   | YEAR | PLACE        | NUMBER | AGE   | METHOD OF HB. DETERMINATIONS  | HB. (GM. PER 100 C.C. BLOOD) | ERYTHROCYTES (MILLIONS PER C.M.M. BLOOD) | PACKED R.B.C. VOLUME (PER CENT) | MEAN CORPUSCULAR VOLUME (C. M. CRONS) | MEAN CORPUSCULAR HB. (MICROGRAMS) | MEAN CORPUSCULAR HB. CONCENTRATION (PER CENT) |
|--|------|--------------|--------|-------|-------------------------------|------------------------------|--|---------------------------------|---------------------------------------|-----------------------------------|---|
| <i>United States of America</i>            |      |              |        |       |                               |                              |  |                                 |                                       |                                   |   |
| Haden <sup>19</sup>                        | 1922 | Kansas City  | 20     | 18-30 | Van Slyke                     | 15.83                        | 5.08                                     |                                 |                                       |                                   |   |
|  |      |              | 20     | 30-50 | Van Slyke                     | 15.23                        | 4.86                                     |                                 |                                       |                                   |   |
| Sackett <sup>20</sup>                      | 1925 | Kansas City  | 15     |       | Wong                          | 16.71                        | 5.09                                     |                                 |                                       |                                   |   |
| Osgood <sup>21</sup>                       | 1926 | Portland     | 137    | 19-30 | Osgood-Haskins                | 15.76                        | 5.39                                     | 44.64                           |                                       |                                   |   |
| Wintrobe and Miller <sup>22</sup>          | 1929 | New Orleans  | 100    | 20-30 | Newcomer                      | 15.87                        | 5.85                                     | 46.50                           |                                       |                                   |   |
| Murphy, Lynch, and Howard <sup>23</sup>    | 1930 | Boston       | 18     |       | Osgood-Haskins                | 15.06                        | 5.35                                     | 44.50                           |                                       |                                   |   |
| Foster and Johnson <sup>24</sup>           | 1931 | New Orleans  | 115    | 18-30 | Van Slyke-Neill               | 15.63                        | 5.26                                     | 44.40                           | 85.92                                 | 30.02                             | 34.77   |
| Osgood, Haskins, and Trotman <sup>25</sup> | 1932 | Portland     | 196    |       | Van Slyke-Neill               | 15.80                        | 5.40                                     | 44.79                           | 87.00                                 | 29.50                             | 35.00   |
| Wintrobe <sup>26</sup>                     | 1932 | New Orleans  |        |       | Osgood-Haskins                |                              |  |                                 |                                       |                                   |   |
| Hadense                                    | 1933 | Cleveland    | 70     |       |                               |                              |  |                                 |                                       |                                   |   |
| Sachs, Levine, and Appels <sup>27</sup>    | 1933 | Omaha        | 100    | 20-25 | Wong                          | 15.34                        | 4.95                                     |                                 |                                       |                                   |   |
| Goldhamer and Fritzel <sup>28</sup>        | 1933 | Omaha        | 100    | 20-25 | Wong                          | 14.93                        | 5.00                                     |                                 |                                       |                                   |   |
| Helmer and Emerson <sup>28</sup>           | 1933 | Ann Arbor    | 100    | 12-17 | Sahli                         | 11.55                        | 4.71                                     |                                 |                                       |                                   |   |
| Brown and Briggs <sup>29</sup>             | 1934 | Indianapolis | 10     | 20-40 | Van Slyke-Neill               | 15.29                        | 5.58                                     |                                 |                                       |                                   |   |
|  | 1934 | St. Louis    | 23     |       | Van Slyke-Neill               | 16.60                        | 5.29                                     | 46.57                           |                                       |                                   |   |
|  |      |              |        |       | protein iron and acid hematin |                              |  |                                 |                                       |                                   |   |
| Walters <sup>30</sup>                      | 1934 | Lawrence     | 100    | 20-30 | Newcomer                      | 15.12                        | 4.84                                     | 46.50                           | 96.50                                 | 31.40                             | 32.40   |
| Walters <sup>31</sup>                      | 1934 | Lawrence     | 10     | 18-25 | Newcomer                      | 16.08                        | 5.26                                     | 49.90                           | 95.00                                 | 30.6                              | 32.10   |
| Osgood <sup>32</sup>                       | 1935 | Portland     | 259    | 14-30 | Osgood-Haskins                | 15.84                        | 5.42                                     | 44.79*                          |                                       |                                   |   |
| Sachs, Levine, and Fabian <sup>30</sup>    | 1935 | Omaha        | 50     | 20-25 | Wong                          | 15.01                        | 4.68                                     |                                 |                                       |                                   |   |
| Peterson and Peterson <sup>31</sup>        | 1935 | Butte        | 75     |       | Dure                          | 86.78                        | 5.17                                     |                                 |                                       |                                   |   |
| Andresen and Muga <sup>32</sup>            | 1936 | Denver       | 40     | 20-45 | Van Slyke-Neill               | 16.54                        | 5.42                                     |                                 |                                       |                                   |   |
| Nelson and Stoker <sup>2</sup>             | 1937 | Lawrence     | 121    | 18-25 | Van Slyke                     | 14.71                        | 5.09                                     | 48.35                           | 89.20                                 | 30.50                             | 34.20   |
|  |      |              | 350    | 18-65 |                               |                              |  |                                 |                                       |                                   |   |
| Mugrage and Andresen <sup>4</sup>          | 1938 | Denver       | 20     | 17-19 | Van Slyke-Neill               | 15.03                        | 5.11                                     | 49.55                           | 92.60                                 | 30.90                             | 33.40   |
|  |      |              | 20     | 19-21 | Van Slyke-Neill               | 16.45                        | 5.35                                     | 48.70                           | 91.00                                 | 30.70                             | 33.80   |
| Myers and Eddy <sup>1</sup>                | 1939 | Cleveland    | 111    |       | Bing and Baker                | 15.66                        |  |                                 |                                       |                                   |   |

|   |                              |   |                                       |                                  |  |  |                      |                |       |       |
|---|------------------------------|---|---------------------------------------|----------------------------------|--|--|----------------------|----------------|-------|-------|
| <i>Europe</i><br>Gram and Norgaard <sup>32</sup>  | 1923                         | Copenhagen  | 10                                    | 20-42                            | Autenrieth-Königsberger Barcroft photometer<br>Van Slyke and spectrophotometer | 15.01<br>15.90<br>14.90<br>14.72                   | 5.45<br>5.06<br>5.07 | 46.54<br>44.00 |       |       |
| Heilmeyer and Hausold <sup>33</sup><br>Bjerring and Sorensen <sup>34</sup><br>Dor <sup>35</sup>   | 1936<br>1936<br>1938         | Jenn<br>Copenhagen<br>Liège   | 80<br>60                              | 18-40                            |  |  |                      |                |       |       |
| <i>South America</i><br>Orin <sup>36</sup>  | 1930                         | Buenos Aires†<br>Buenos Aires†<br>Jujuy†<br>Mendoza†<br>Corrientes†<br>Buenos Aires | 321<br>82<br>261<br>260<br>262<br>50  |                                  | Newcomer<br>Newcomer<br>Newcomer<br>Newcomer<br>Newcomer                       | 14.28<br>15.30<br>14.55<br>14.56<br>14.49<br>14.80 | 5.30                 | 43.17          |       |       |
| Tenconi <sup>37</sup>   | 1931                         |   |                                       |                                  |  |  |                      |                |       |       |
| <i>Australia</i><br>Wardlaw, Barry, McDonald,<br>and McIntyre <sup>38</sup>   | 1935                         | Sidney<br>Central Australia<br>Aborigines   | 26<br>13<br>25                        |                                  | Newcomer<br>Newcomer<br>Newcomer   | 15.83<br>14.95<br>15.11                            |                      |                |       |       |
| <i>Philippines</i><br>Chamberlain <sup>39</sup><br>Navarro <sup>40</sup>  | 1911<br>1937                 | Manila (soldiers)<br>Manila   | 702<br>104                            | 19-39                            | Dare and von Fleischel<br>Wong   | 89.60%<br>14.11                                    | 5.20<br>5.16         | 43.00          | 27.00 | 32.00 |
| <i>India</i><br>Sodkey, Goldale, Malankar,<br>and Billimoria <sup>41</sup><br>Shourie and Singh <sup>42</sup><br>Sankaran and Rajagopal <sup>43</sup><br>Cropper and Kalath <sup>44</sup> | 1937<br>1938<br>1938<br>1938 | Bombay<br>Wellington<br>Madras<br>Burmese<br>Karens                                 | 121<br>10<br>125<br>Burmese<br>Karens | 19-30<br>20-40<br>18-25<br>18-45 | Van Slyke-Neill<br>Bing and Baker<br>Bing and Baker<br>Hellige                 | 15.37<br>20.92<br>16.57<br>14.38<br>14.09          | 5.11                 | 41.72          |       |       |
| Napier and Majumdar <sup>45</sup>   | 1938                         | Chleutna  | 25                                    | 20-40                            | Hellige  | 12.00  | 5.05                 | 42.18          | 25.14 | 29.72 |
| <i>Africa</i><br>Lakuntzky <sup>46</sup>  | 1934                         | Johannesburg  | 60                                    |                                  | Haldane-Gower  | 14.54  | 5.99                 |                |       |       |
| <i>Present study</i>  | 1939                         | Honolulu  | 137                                   | 16-25                            | Acid-hematin photo-<br>electric colorimeter                                    | 15.10  | 5.08                 | 44.20          | 29.20 | 34.70 |

\*Cell volume for 153 men 20 to 30 years of age.

†Conscripts.

‡Students.

studies of normal values for platelets. The results of studies of normal values for the various blood elements for men in the literature available to us are summarized in Tables I and II for the purpose of comparison with the results of investigations of mean blood values presented here.

#### SUBJECTS EXAMINED

The 137 men examined in this study were all university students, ranging in age from 16 to 25 years. All had resided in the Hawaiian Islands for at least two years, and the greater number of them were born there and had lived continuously in the Islands. The individuals were all of average good health, and none had had recent illness. They were of different racial groups, the number of persons in each age group, and as well the number in each racial group, are indicated in Tables III and IV. Honolulu is situated on the seashore, and the homes of its residents range from sea level to an altitude of approximately 1,000 feet.

#### METHODS OF STUDY

All blood samples of the subjects examined were of capillary blood, obtained from a deep puncture of the finger made with a sharp spring lancet, only the freely flowing blood being used. The hemoglobin estimations for 31 men were made by the acid hematin method, employing a Bausch & Lomb colorimeter equipped with a standard hemoglobin color disk. The color disk was checked for accuracy during the course of the study. The remaining hemoglobin estimations were made with a Klett-Summerson photoelectric colorimeter employing the acid hematin method, the colorimeter being checked and standardized against the Van Slyke oxygen capacity method of hemoglobin determination.

Enumerations of erythrocytes, leucocytes, and platelets were made by the use of Thoma pipettes and Levy counting chambers, certified for accuracy by the United States Bureau of Standards. Hayem's solution and Tuerk's solution were used in diluting the blood samples for red and white blood cell counts made in the usual manner. The solution of Rees and Ecker was used for dilution of blood samples for platelets, the counts being made by the direct method. The volume of packed red blood cells was determined by the use of capillary hematocrit pipettes according to the method described by one of us (C. J. H.),<sup>10</sup> and calculations of the corpuscular constants were made by the use of the formulas suggested by Wintrobe.<sup>11</sup> Differential leucocyte counts were made from blood smears stained with Wright's stain and counterstained with Giemsa. Counts were made from three smears for each person, the average for the three counts being taken as the differential count for the person.

The data obtained in this study were submitted to statistical analysis, and mean values were determined for each blood element for each age group and racial group. Statistical constants were calculated in all instances. The results and conclusions presented in this paper are based on the statistical studies of these data.

#### OBSERVATIONS AND RESULTS

It has frequently been suggested that values for the different blood elements may vary for different racial groups. It has also been suggested that



TABLE II  
MEAN VALUES FOR PLATELETS, LEUCOCYTES, AND DIFFERENTIAL LEUCOCYTE COUNTS REPORTED FOR MEN AND FOR MEN AND WOMEN

| MEAN VALUES FOR PLATELETS, LEUCOCYTES, AND DIFFERENTIAL LEUCOCYTE COUNTS REPORTED FOR MEN AND FOR MEN AND WOMEN |              |                    |       |       |  |                               |  |              |              |            |             |           |
|---|--------------|--------------------|-------|-------|--|-------------------------------|--|--------------|--------------|------------|-------------|-----------|
| YEAR  | PLACE        | NUMBER OF SUBJECTS | AGE   | SEX   | PLATELETS (THOUSANDS PER C.M.M. BLOOD) | LEUCOCYTES (PER C.M.M. BLOOD) | DIFFERENTIAL LEUCOCYTE COUNTS (PER CENT) |              |              |            |             |           |
|   |              |                    |       |       |  |                               | NEUTROPHILES                             | "STAB" CELLS | EOSINOPHILES | BASOPHILES | LYMPHOCYTES | MONOCYTES |
| 1923  | New York     | 80                 |       |       |  | 7,379                         | 51.04                                    |              | 2.31         | 0.85       | 36.04       | 9.62      |
| 1927  | Boston       |                    |       |       |  | 2,700-14,000<br>6,309         |  |              |              |            |             |           |
| 1929  |              |                    |       |       |  | 6,000-9,000                   | 50.64                                    |              | 1.30         | 0.51       | 25.39       | 6.9       |
| 1933  | Johannesburg | 171                | 20-25 |       |  | 7,559                         | 54.20                                    |              | 1.88         | 0.69       | 39.72       | 4.24      |
| 1933  | Omaha        | 100                |       |       |  | 4,700-11,500<br>6,280         |  |              |              |            |             |           |
| 1933  |              |                    |       |       |  | 8,350                         |  |              |              |            |             |           |
| 1933  | Butte        | 75                 |       | M     |  | 8,260                         | 54.36                                    |              | 2.00         | 0.60       | 36.26       | 6.33      |
| 1935  |              | 100                |       | M & F |  | 5,618                         |  |              |              |            |             |           |
| 1935  | Nashville    | 18                 |       | M     |  | 7,447                         |  |              |              |            |             |           |
| 1935  | Portland     | 269                | 19-30 | M & F |  |                               | 54.26                                    | 0.75         | 1.90         |            | 37.76       | 4.18      |
| 1935  |              | 378                | 14-30 | M & F |  |                               |  |              |              |            |             |           |
|   |              | 236                | 20-30 | M & F |  |                               |  |              |              |            |             |           |
|   |              | 597                | 4-30  | M & F |  |                               | 52.68                                    | 0.13         | 3.9          | 0.0        | 1.90        | 0.6-7.0   |
| 1937  | Calcutta     | 6                  | 20-40 | M     |  | 7,111                         |  |              |              |            |             |           |
| 1938  | Portland     | 25                 | 15-18 | M     |  | 8,220                         | 43.30                                    | 1.00         | 2.30         | 0.80       | 44.10       | 4.20      |
| 1939  |              | 41                 | 15-18 | M     |  | 8,190                         | 46.30                                    | 1.00         | 2.10         | 0.70       | 42.20       | 4.30      |
|   |              | 87                 | 15-18 | M & F |  | 7,300                         | 53.20                                    | 0.60         | 2.00         | 0.50       | 38.50       | 4.20      |
| 1939  | Portland     | 198                | 19-38 | M     |  | 7,350                         | 53.90                                    | 0.70         | 1.80         | 0.50       | 38.10       | 4.20      |
|   |              | 269                | 19-38 | M & F |  |                               |  |              |              |            |             |           |
| 1901  | Boston       |                    |       | M     | 778,000-862,000*                       |                               |  |              |              |            |             |           |
| 1932  |              |                    |       |       | 500,000-900,000*                       |                               |  |              |              |            |             |           |
|   |              |                    |       |       | 716,000                                |                               |  |              |              |            |             |           |
| 1933  | London       | 11                 |       |       | 590,000-760,000*                       |                               |  |              |              |            |             |           |
| 1935  | Boston       | 22                 |       | M     | 525,000*                               |                               |  |              |              |            |             |           |
| 1921  |              |                    |       |       | 284,000†                               |                               |  |              |              |            |             |           |
| 1929  |              |                    |       |       | 536,000†                               |                               |  |              |              |            |             |           |
| 1939  | Honolulu     | 137                | 16-25 | M     | 253,000†                               | 7,067                         | 54.63                                    |              | 2.93         | 0.57       | 36.81       | 5.08      |
|   |              |                    |       |       |  |                               |  |              |              |            |             |           |

\*Indirect method of platelet enumeration.

†Direct method of platelet enumeration.

values for different geographical regions may vary due to operation of factors of climate and altitude. Age differences for adult men, however, are thought not to occur, and if they do they may be so slight as to be insignificant. The extensive data collected in this investigation were analyzed to determine whether age or racial differences occurred for this series of persons. If age and racial differences were found not to occur, mean values could be calculated for all the blood elements for the entire group, these values to serve as standard blood values for the Hawaiian Islands for comparison with blood values reported for other geographical regions of the world.

The persons examined in this study ranged in age from 16 to 25 years, but the number in each age group was great enough to permit critical analysis only for the ages from 18 to 22 inclusive. General observations of the blood values for these age groups, supported by statistical analysis, showed that significant differences in the blood values did not occur. The values for the various blood elements for the different ages are summarized in Table III.

In the study of racial differences in blood values, sufficiently large numbers of persons to permit statistical analysis occurred only for the Caucasian, Chinese, and Japanese racial groups. The number of persons of other racial groups attending the University of Hawaii is so small that many years would be required for critical studies. For the three larger racial groups, statistically significant differences for none of the blood elements occurred. The values for the various blood elements for the different racial groups are summarized in Table IV.

Since significant age and racial differences for the various blood elements did not occur, all these data were combined for determination of normal blood values for young men residing in the Hawaiian Islands. The mean values, statistical constants, and the range of the values for the different blood elements are presented in Table V. The frequency of occurrence of the various blood values is graphically presented in histograms in Chart 1.

The mean values for the various blood elements obtained in this study for healthy young men of the Hawaiian Islands may be compared to those for men of other geographical regions summarized in Tables I and II. Such a comparison indicates that the values obtained in this study are closely similar to those given by the greater number of investigators. It is evident that particular or peculiar types of normal blood values do not exist for adult men of the Hawaiian Islands.

#### DISCUSSION.

It is generally agreed that blood values for healthy adult males are constant. This feature of blood values has been pointed out by Osgood,<sup>6</sup> Andresen and Mugrage,<sup>3</sup> Wintrobe,<sup>12</sup> and many others. Our conclusion that age differences did not occur for the blood values of the series of healthy persons examined in this study was, therefore, anticipated, particularly in view of the narrow age limits of the group examined. Also, it has been pointed out by Osgood,<sup>6</sup> Mugrage and Andresen,<sup>4</sup> Sachs, Levine, and Griffith,<sup>13</sup> Goldhamer and Fritzell,<sup>14</sup> and many others that adult blood values are attained by males between the ages of 15 and 17 years. Our data tend to support these observations.

TABLE III  
COMPARISON OF AVERAGE BLOOD VALUES OF DIFFERENT AGE GROUPS

| AGE   | NUMBER OF SUBJECTS | AVERAGE HB. (GM. PER 100 C.C. BLOOD) | AVERAGE NUMBER ERYTHROCYTES (MILLIONS PER C.MM. BLOOD) | AVERAGE VOLUME PACKED R.B.C. (C.C. PER 100 C.C. BLOOD*) | MEAN CORPUSCULAR VOLUME (C. MICRONS) | MEAN CORPUSCULAR HGB. (MICROGRAMS) | MEAN CORPUSCULAR HGB. CONCENTRATION (PER CENT) |
|-------|--------------------|--------------------------------------|--|---|--------------------------------------|------------------------------------|--|
| 16    | 1                  | 13.01                                | 4,540,000  | 41.7  | 91.4                                 | 28.3                               | 31.2   |
| 17    | 4                  | 15.51                                | 5,390,000  | 43.8  | 82.7                                 | 28.6                               | 35.0   |
| 18    | 13                 | 14.95                                | 5,009,000  | 42.0  | 84.9                                 | 29.7                               | 35.2   |
| 19    | 35                 | 15.22                                | 5,139,000  | 44.9  | 88.2                                 | 29.7                               | 34.2   |
| 20    | 34                 | 15.09                                | 4,997,000  | 44.4  | 87.6                                 | 30.0                               | 35.0   |
| 21    | 23                 | 14.92                                | 5,057,000  | 43.7  | 86.1                                 | 29.3                               | 34.7   |
| 22    | 13                 | 14.74                                | 5,201,000  | 44.5  | 85.5                                 | 28.6                               | 33.7   |
| 23    | 7                  | 15.22                                | 5,187,000  | 43.9  | 84.6                                 | 29.2                               | 34.5   |
| 24    | 3                  | 15.65                                | 5,153,000  | 45.4  | 83.3                                 | 29.5                               | 36.3   |
| 25    | 4                  | 15.92                                | 4,936,000  | 44.7  | 88.3                                 | 30.7                               | 35.5   |
| 10-25 | 137                | 15.10                                | 5,082,000  | 44.2  | 86.5                                 | 29.2                               | 34.7   |

\*Cell volume and corpuscular constants determined for 107 subjects.

| AGE   | NUMBER OF SUBJECTS | AVERAGE NUMBER PLATELETS (PER C.MM. BLOOD**) | AVERAGE NUMBER LEUCOCYTES (PER C.MM. BLOOD) | AVERAGE DIFFERENTIAL LEUCOCYTE COUNTS (PER CENT) |              |            |             |           |
|-------|--------------------|--|---|--|--------------|------------|-------------|-----------|
|       |                    |  |   | NEUTROPHILES                                     | EOSINOPHILES | BASOPHILES | LYMPHOCYTES | MONOCYTES |
| 16    | 1                  | 245,000                                      | 7,550                                       | 60.00  | 1.34         | 0.67       | 33.67       | 4.00      |
| 17    | 4                  | 252,000                                      | 6,600                                       | 55.42  | 4.17         | 0.42       | 34.08       | 5.84      |
| 18    | 13                 | 253,000                                      | 7,270                                       | 51.31  | 3.64         | 0.74       | 38.72       | 5.59      |
| 19    | 35                 | 201,000                                      | 6,700                                       | 54.65  | 2.90         | 0.57       | 36.55       | 5.48      |
| 20    | 34                 | 256,000                                      | 7,250                                       | 55.42  | 2.57         | 0.49       | 36.62       | 4.70      |
| 21    | 23                 | 248,000                                      | 6,820                                       | 56.32  | 3.11         | 0.52       | 34.97       | 5.06      |
| 22    | 13                 | 251,000                                      | 7,450                                       | 57.51  | 2.81         | 0.70       | 34.08       | 4.97      |
| 23    | 7                  | 233,000                                      | 6,090                                       | 52.09  | 2.53         | 0.76       | 40.48       | 4.34      |
| 24    | 3                  | 239,000                                      | 6,463                                       | 52.22  | 2.45         | 0.78       | 39.89       | 4.45      |
| 25    | 3                  | 269,000                                      | 6,140                                       | 50.00  | 5.34         | 0.34       | 40.34       | 4.00      |
| 10-25 | 136                | 253,000                                      | 7,067                                       | 54.63  | 2.93         | 0.57       | 36.81       | 5.08      |

\*\*Platelets determined for 137 subjects.

TABLE IV  
COMPARISON OF AVERAGE BLOOD VALUES OF DIFFERENT RACIAL GROUPS

| RACE               | NUMBER OF SUBJECTS | AVERAGE HB. (GM. PER 100 C.C. BLOOD) | AVERAGE NUMBER ERYTHROCYTES (MILLIONS PER C.MM. BLOOD) | AVERAGE VOLUME OF PACKED R.B.C. (C.C. PER 100 C.C. BLOOD*) | MEAN CORPUSCULAR VOLUME (C. MICRONS) | MEAN CORPUSCULAR HB. (MICROMICROGRAMS) | MEAN CORPUSCULAR HB. CONCENTRATION (PER CENT) |
|--------------------|--------------------|--------------------------------------|--|--|--------------------------------------|--|---|
| Caucasian          | 40                 | 15.04                                | 5,027,000  | 42.0   | 84.5                                 | 29.5                                   | 35.3  |
| Chinese            | 39                 | 15.10                                | 5,158,000  | 45.1   | 87.0                                 | 28.4                                   | 33.9  |
| Japanese           | 39                 | 15.23                                | 5,084,000  | 44.9   | 88.3                                 | 29.6                                   | 34.8  |
| Korean             | 10                 | 15.30                                | 5,008,000  | 44.5   | 88.5                                 | 30.3                                   | 34.5  |
| Caucasian-Hawaiian | 8                  | 14.62                                | 5,049,000  | 43.3   | 85.4                                 | 28.8                                   | 34.6  |
| Chinese-Hawaiian   | 1                  | 16.60                                | 5,110,000  | 44.6   | 87.1                                 | 32.3                                   | 37.2  |
| All individuals    | 137                | 15.10                                | 5,082,000  | 44.2   | 86.5                                 | 29.2                                   | 34.7  |

\*Cell volume and corpuscular constants determined for 107 subjects.

| RACE               | NUMBER OF SUBJECTS | AVERAGE NUMBER PLATELETS (PER C.MM. BLOOD†) | AVERAGE NUMBER LEUCOCYTES (PER C.MM. BLOOD) | AVERAGE DIFFERENTIAL LEUCOCYTE COUNT (PER CENT) |              |             |            |           |
|--------------------|--------------------|---|---|---|--------------|-------------|------------|-----------|
|                    |                    |   |   | NEUTROPHILES                                    | EOSINOPHILES | LYMPHOCYTES | BASOPHILES | MONOCYTES |
| Caucasian          | 39                 | 262,000                                     | 7,970                                       | 53.90   | 3.53         | 37.57       | 0.68       | 5.22      |
| Chinese            | 39                 | 247,000                                     | 7,090                                       | 53.97   | 2.57         | 37.69       | 0.52       | 5.12      |
| Japanese           | 39                 | 248,000                                     | 7,120                                       | 55.01   | 3.08         | 35.53       | 0.53       | 5.08      |
| Korean             | 10                 | 236,000                                     | 6,350                                       | 59.60   | 1.50         | 33.94       | 0.63       | 4.37      |
| Caucasian-Hawaiian | 8                  | 293,000                                     | 6,620                                       | 52.88   | 2.71         | 39.29       | 0.42       | 5.00      |
| Chinese-Hawaiian   | 1                  | 233,000                                     | 7,000                                       | 58.67   | 2.34         | 32.34       | 0.00       | 6.34      |
| All individuals    | 136                | 253,000                                     | 7,067                                       | 54.63   | 2.93         | 36.81       | 0.57       | 5.08      |

†Platelets determined for 137 subjects.

It has frequently been suggested that racial differences for blood values may occur and that these variations may account for differences in mean blood values reported by different authors. It is, however, a problem on which very little direct work has been undertaken. Hamre and Wong,<sup>15</sup> in a study of normal blood values for pre-school age children of different races, particularly Chinese, Japanese, and Caucasian, found the blood values to be similar, with

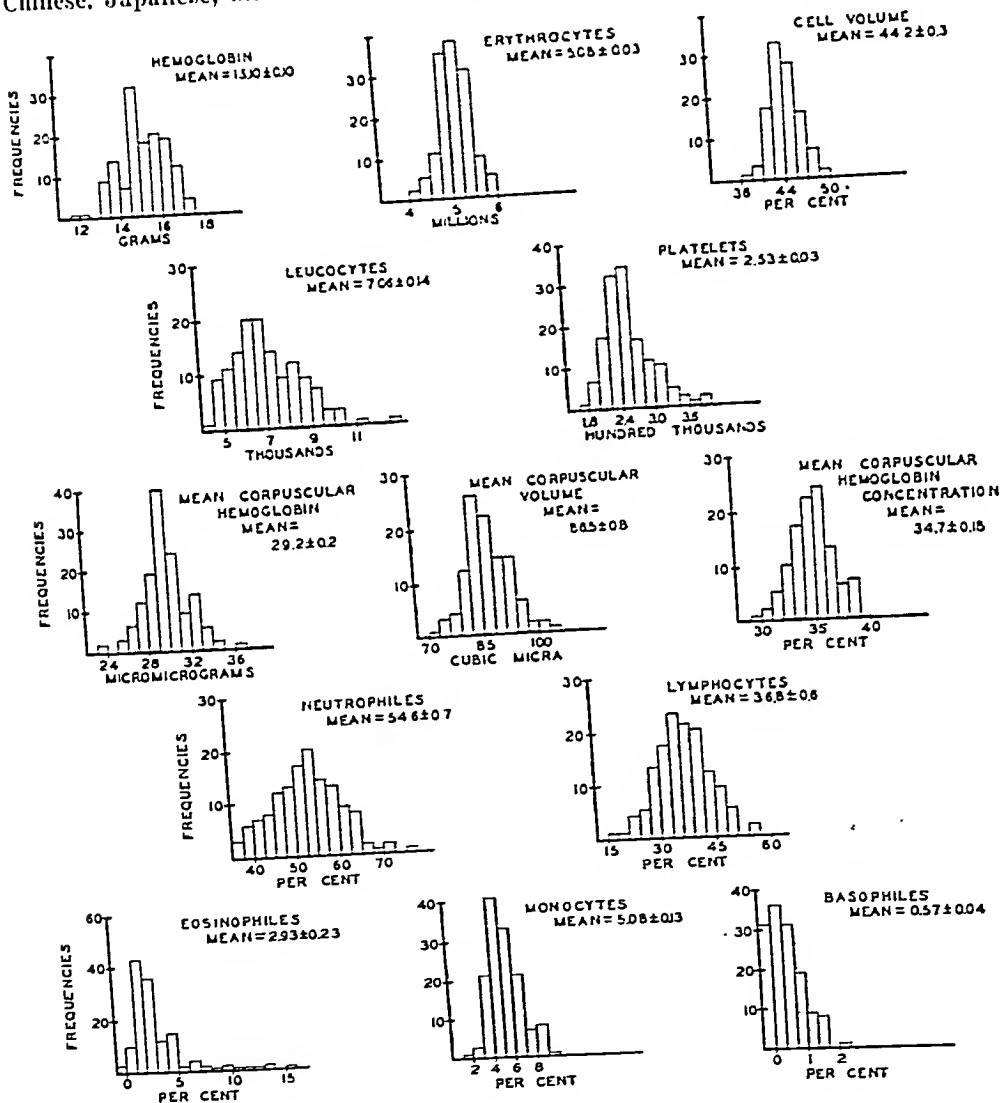


Chart 1.

no significant differences. Nelson and Stoker,<sup>2</sup> in studies of hemoglobin and red blood cell values of white and negro adult men, found no racial differences to exist. Myers and Eddy,<sup>1</sup> after a study of hemoglobin values reported in the literature for different races, came to the conclusion that racial differences did not occur. A study of Tables I and II, which include blood values for several races, indicates that, when due consideration is given to variations in technique

employed in the studies, racial differences in blood values do not exist, or if they exist, they are very small. The present study supports these conclusions, since it shows that for Chinese, Japanese, and Caucasian men of similar ages and environment, statistically significant differences for the mean values of none of the blood elements occur.

It has also frequently been suggested that the differences in mean blood values reported by different investigators may be due to geographical factors other than that of altitude. Walters,<sup>16, 17</sup> Wintrobe,<sup>12</sup> and Myers and Eddy<sup>1</sup> have concluded that significant differences of blood values for different geographical regions do not exist, and that blood values are generally the same for all regions of the world. The mean values reported by various investigators from various sections of the world, presented in Tables I and II, indicate that this conclusion must be accepted, and that the normal blood values for men residing in the Hawaiian Islands cannot be considered to differ from the blood values of men residing in other regions of the world.

Mean blood values for normal persons, as Nelson<sup>18</sup> and others have pointed out, represent only the central values of normal, the normal values ranging above and below these means. Mean values, however, may be employed in making comparisons for the degree of similarity and agreement of the results of different investigations. The mean values for hemoglobin, obtained by different investigators and presented in Table I, vary greatly though the greater number fall in the region of 14.5 to 15.5 Gm. per 100 c.c. of blood. Since various methods were used in making the hemoglobin determinations, it is possible that the variation in mean values obtained may be due to a great extent to the differences in methods employed. Where the technique and apparatus employed have been quite uniform, as for instance in the enumeration of red blood cells, the results of the different investigations are more nearly alike. The greater number of red blood cell values summarized in Table I fall into the interval of 5 to 5.5 millions per cubic millimeter of blood. Our mean hemoglobin value of 15.10 Gm. and mean number of red blood cells of 5.08 millions, therefore, fall in the regions which include the greater number of mean values reported for these blood elements.

The mean values for the volume of packed red blood cells of healthy adult men reported in the literature vary markedly. This is undoubtedly due to a great extent to differences in technique employed in the determinations. Other factors may also contribute to this difference in reported values. A number of recent workers (Walters,<sup>17</sup> Andresen and Mugrage,<sup>3</sup> and Mugrage and Andresen<sup>4</sup>) have reported mean values of 48 and 49 per cent for cell volume. These values are exceptional, the greater number of investigators having reported values ranging from 44 to 46 per cent. Our mean values of 44.2 per cent for the volume of packed red blood cells, therefore, fall in the group of more frequently reported values.

The corpuscular constants have received only little consideration except in recent investigations. The mean values for mean corpuscular hemoglobin concentration tend to be nearly alike and fall in the interval 32 to 35 per cent. Values for mean corpuscular hemoglobin are also in general agreement, and the greater number of mean values vary from 29 to 31 micromicrograms. Greater differences for mean corpuscular volume reported in the literature

occur and vary from 84 to 96 cubic microns. The same investigators who report high mean values for packed red blood cell volume have also reported high values for mean corpuscular volume. Our values of 34.7 per cent for mean corpuscular hemoglobin concentration, 29.2 micromicrograms for mean corpuscular hemoglobin, and 86.5 cubic microns for mean corpuscular volume agree with the values reported by the greater number of investigators and with the values of 35 per cent, 29.5 micromicrograms, and 87 cubic microns originally suggested by Wintrobe.<sup>11</sup>

TABLE V

MEAN VALUES, STATISTICAL CONSTANTS, AND RANGE OF VALUES FOR ENTIRE GROUP\*

| BLOOD ELEMENT   | MEAN<br>VALUE | STANDARD<br>ERROR OF<br>THE MEAN | STANDARD<br>DEVIATION | RANGE          |
|---|---------------|----------------------------------|-----------------------|----------------|
| Hemoglobin in grams per 100 c.c. of blood                                   | 15.10         | 0.10                             | 1.11                  | 11.83 to 17.20 |
| Erythrocytes in millions per cubic millimeter of blood                      | 5.08          | 0.03                             | 0.35                  | 4.23 to 5.97   |
| Volume of packed red blood cells in cubic centimeters per 100 c.c. of blood | 44.2          | 0.3                              | 2.8                   | 37.5 to 51.6   |
| Mean corpuscular volume in cubic microns                                    | 86.5          | 0.8                              | 7.9                   | 71.2 to 103.2  |
| Mean corpuscular hemoglobin in micromicrograms                              | 29.2          | 0.2                              | 2.2                   | 23.1 to 36.1   |
| Mean corpuscular hemoglobin concentration in per cent                       | 34.7          | 0.18                             | 1.7                   | 29.9 to 38.9   |
| Platelets in ten thousands per cubic millimeter of blood                    | 25.3          | 0.03                             | 0.40                  | 17.8 to 39.8   |
| Leucocytes in thousands per cubic millimeter of blood                       | 7.06          | 0.14                             | 1.56                  | 4.4 to 12.6    |
| Differential leucocyte count in per cent                                    |               |                                  |                       |                |
| Neutrophiles  | 54.6          | 0.70                             | 8.0                   | 38.6 to 78.3   |
| Eosinophiles  | 2.93          | 0.23                             | 2.7                   | 0.0 to 15.6    |
| Basophiles  | 0.57          | 0.04                             | 0.48                  | 0.0 to 2.2     |
| Lymphocytes   | 36.81         | 0.6                              | 6.9                   | 16.3 to 55.6   |
| Monocytes   | 5.08          | 0.12                             | 1.47                  | 1.6 to 9.2     |

\*All mean values determined for 127 persons, except for leucocytes and differential leucocyte counts, which are for 136 persons, and cell volume and corpuscular constants for 107 persons.

The mean values reported for platelets may be divided into two groups, those obtained by the direct method and those obtained by the indirect method of enumeration. The mean values obtained by the two methods have been reviewed by Olef.<sup>9</sup> In general, the values obtained by the first method are about half as great as those obtained by the second method, and range from 250,000 to 300,000 per cubic millimeter of blood. We chose the direct method of enumerating platelets, and our mean value of 253,000 per cubic millimeter of blood compares well with the mean values obtained by this method by other investigators.

The mean values for the normal number of white blood cells reported in the literature available to us, and summarized in Table II, vary from 5,618 to 8,350 per cubic millimeter of blood. Our mean value of 7,067 falls well within the limits of these observed values. The mean values of 54.6 per cent for neutrophiles, 2.93 per cent for eosinophiles, 0.57 per cent for basophiles, 36.81 per cent for lymphocytes, and 5.08 per cent for monocytes obtained in this study compare closely to the mean values obtained by Osgood,<sup>6</sup> and Osgood

and co-workers.<sup>7, 8</sup> They, however, differ from the values usually given in textbooks in that the mean values for neutrophils are lower while the mean values for lymphocytes are higher than values commonly considered to be normal.

#### SUMMARY

Data are presented for the blood values of 137 healthy young men residing in the Hawaiian Islands, and ranging in age from 16 to 25 years. They were of several different races, the larger number being Caucasians, Chinese, and Japanese. In this study, age or race differences were not found to occur for values for any of the various blood elements. The mean blood values obtained compared closely with those given by investigators in other parts of the world, and we have concluded that blood values for men in the Hawaiian Islands do not differ significantly from those of men residing elsewhere. The mean values, statistical constants, and range of observed values for the various blood elements are summarized in Table V.

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## THE AGGLUTININ RESPONSE OF NORMAL PERSONS TO SKIN TESTS WITH BRUCELLERGEN AND BRUCELLA VACCINE\*

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TWO types of antigens are commonly employed in skin testing for brucellosis. One, Huddleson's brucellergen, contains the purified protein nucleate fraction of *Brucella* cells. The other, *Brucella* vaccine, is a suspension of heat-killed organisms in physiologic saline, and is also used for therapy.

There is evidence that the intradermal injection of these substances stimulates the production of agglutinins. Giordano,<sup>1</sup> Goldstein,<sup>2</sup> Heathman,<sup>3</sup> and Meyer and Eddie,<sup>4</sup> have noted positive agglutination tests following skin tests with heat-killed organisms. Wide variations are reported, however, in the maximum titers obtained. For example, Goldstein recorded titers as high as 1:5,120, while the highest titer noted by Meyer was 1:80. Similar differences have occurred with brucellergen. Evans<sup>5</sup> found titers as high as 1:320, while Huddleson<sup>6</sup> states that "one skin injection of brucellergen may give rise to *Brucella* agglutinins in a low titer in a small percentage of individuals. The agglutinins disappear in about sixty days."

Among the factors contributing to these variations are (1) individual differences in agglutinin response, (2) the size of the skin test dose, (3) the nature of the antigen used for the agglutination tests, and (4) the technique of reading the agglutination tests. Since no single observer has studied both brucellergen and vaccine at the same time, it is difficult to draw conclusions as to their relative ability to stimulate agglutinin production.

Since this development of agglutinins may cause difficulty in interpreting agglutination tests, it is the routine procedure in some clinics to take blood for the agglutination test before the skin test is performed. However, since the results have been variable, as described, and since there is considerable difference of opinion concerning the relative merits of the skin and agglutination tests,

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there is no general agreement on this point. For example, physicians using Huddleson's brucellergen are advised that "the first procedure to follow in arriving at a diagnosis is the performing of an intradermal test with brucellergen." Thus, in a clinic where patients are usually seen after consulting one or more other physicians, we are constantly faced with the problem of interpreting agglutination tests performed at intervals of several days to several weeks following skin tests, and it is of considerable importance to know as accurately as possible the effect of the skin test itself.

Due to marked local and systemic reactions in sensitive individuals, the size of the skin test dose has been reduced in the past few years, both for brucellergen and for the heat-killed organisms. Keller<sup>7</sup> has shown that the smaller doses of heat-killed organisms are equally as reliable as brucellergen for skin testing, and that reducing the size of the dose has abolished the more severe reactions previously noted.

Our purpose in undertaking this study was to determine the agglutinin response to commercial products commonly used at the present time, hoping we might, with these smaller doses, obtain information of value in interpreting agglutination tests performed on patients at varying intervals following skin tests. It also seemed of interest to compare the effects of vaccine and brucellergen, using the same antigen and the same technique in interpreting the tests.

#### METHOD AND MATERIALS

Fifty apparently normal, healthy adults, most of them members of the house staff of Stanford University Hospitals, volunteered for the study. All had negative *Brucella* agglutinations before being skin tested. One-half were given 0.1 c.c. of Huddleson's brucellergen 1:12,000, obtained from the Department of Bacteriology of the Michigan State College, and the other one-half were given 0.1 c.c. of the commercial *Brucella* vaccine, prepared by the Lederle Laboratories, diluted so that 0.1 c.c. contained forty million heat-killed organisms. The skin tests were read after forty-eight hours, an area of induration and edema of 5 mm. or more being considered positive. Agglutination tests were performed during the second and fourth weeks following the skin tests.

The antigen was prepared from *Br. abortus* strain 19, from which contaminants and rough variants were carefully excluded. A forty-eight-hour growth of the organisms was suspended in 0.5 per cent phenolized saline, and diluted to a density corresponding to approximately 4 cm. on the Gates apparatus. In performing the tube agglutination tests, serial dilutions of the serum, from 1:10 to 1:160, were added to constant amounts of the antigen. The tests were read after twelve hours' incubation at 37° C., and again after standing at room temperature for twenty-four hours. Tubes not showing a perfectly clear supernatant solution, with good clumping in the bottom, were regarded as negative. Tube agglutination tests were also performed on many sera with the stock antigen, prepared by the United States Department of Agriculture, as a check on the sensitivity of our own antigen. Finally, rapid slide agglutination tests were performed, using commercial *Br. abortus* febrile antigen, prepared by the Lederle Laboratories. The dilutions ranged from 1:20 to 1:160. These were read after standing ten minutes.

## RESULTS

The results are presented in the accompanying tables. Individual skin and agglutination tests are recorded in Tables I and II, while Table III is a comparison of the results obtained with the two antigens. For convenience, Tables I and II are arranged in the following order: positive skin and agglutination tests, negative skin and positive agglutination tests, positive skin and negative agglutination tests, and negative skin and negative agglutination tests.

TABLE I  
RESULTS IN PERSONS SKIN TESTED WITH BRUCELLERGEN

| NUMBER | SKIN TEST | AGGLUTINATION TEST<br>SECOND WEEK AFTER<br>SKIN TEST |      | AGGLUTINATION TEST<br>FOURTH WEEK AFTER<br>SKIN TEST |      |
|--------|-----------|--|------|--|------|
|        |           | SLIDE  | TUBE | SLIDE  | TUBE |
| 1      | +         | 0  | 1:10 | 0  | 1:10 |
| 2      | +         | 1:40   | 1:40 | 1:20   | 1:20 |
| 3      | +         | 1:80   | 1:40 | 1:40   | 1:40 |
| 4      | +         | 0  | 1:20 | 1:20   | 1:20 |
| 5      | +         | 1:20   | 1:20 | 1:20   | 1:20 |
| 6      | +         | 0  | 0    | 0  | 1:20 |
| 7      | +         | 1:20   | 1:40 | 1:40   | 1:40 |
| 8      | +         | 0  | 1:10 | 1:20   | 1:20 |
| 9      | +         | 1:80   | 1:80 | 1:40   | 1:40 |
| 10     | -         | 1:20   | 1:40 | 1:40   | 1:40 |
| 11     | -         | 1:80   | 1:80 | 1:40   | 1:80 |
| 12     | -         | 1:40   | 1:40 | 1:40   | 1:40 |
| 13     | -         | 0  | 1:20 | 0  | 1:20 |
| 14-15  | +         | 0  | 0    | 0  | 0    |
| 16-25  | -         | 0  | 0    | 0  | 0    |

TABLE II  
RESULTS OF PERSONS SKIN TESTED WITH BRUCELLA VACCINE

| NUMBER | SKIN TEST | AGGLUTINATION TEST<br>SECOND WEEK AFTER<br>SKIN TEST |      | AGGLUTINATION TEST<br>FOURTH WEEK AFTER<br>SKIN TEST |      |
|--------|-----------|--|------|--|------|
|        |           | SLIDE  | TUBE | SLIDE  | TUBE |
| 1      | +         | 0  | 1:20 | 1:20   | 1:20 |
| 2      | +         | 0  | 1:10 | 0  | 1:10 |
| 3      | +         | 1:80   | 1:80 | 1:80   | 1:40 |
| 4      | +         | 1:40   | 1:40 | 1:20   | 1:20 |
| 5      | +         | 1:20   | 1:20 | 1:20   | 1:20 |
| 6      | +         | 1:40   | 1:40 | 1:40   | 1:20 |
| 7      | -         | 0  | 1:10 | 0  | 0    |
| 8      | -         | 1:20   | 1:20 | 1:20   | 1:20 |
| 9      | -         | 0  | 1:10 | 0  | 1:10 |
| 10     | -         | 1:20   | 1:20 | 0  | 1:10 |
| 11     | -         | 0  | 1:10 | 0  | 1:20 |
| 12     | -         | 1:40   | 1:40 | 1:40   | 1:40 |
| 13     | +         | 0  | 0    | 0  | 0    |
| 14-25  | -         | 0  | 0    | 0  | 0    |

Eleven of the group tested with brucellergen and 7 of the vaccine group developed positive intradermal reactions. Five of the former and three of the latter developed red streaks up the arm, with swollen, painful axillary nodes. General malaise, chills, and fever occurred in two instances. In spite of these strongly positive reactions no local tissue necrosis resulted. A mild secondary infection of the skin developed in one instance. The local induration and edema

tended to be firmer and deeper following vaccine than following brucellergen, and persisted almost a month in two cases.

In each group about one-half of the persons failed to develop agglutinins. In the others the titers were uniformly low. The maximum of 1:80 was attained in only four instances. All but three with positive skin tests developed agglutinins. Of those with negative intradermal reactions, four in the brucellergen and six in the vaccine group also developed agglutinins, and the titers were equally as high as in those in whom the skin test was positive. From Table III it is apparent that there was very little, if any, difference in the response to the two skin test antigens.

TABLE III  
RESULTS GROUPED ACCORDING TO THE MAXIMUM TITERS ATTAINED

| SKIN TEST ANTIGEN | NUMBER OF POSITIVE SKIN TESTS | 0  | 1:10 | 1:20 | 1:40 | 1:80 |
|-------------------|-------------------------------|----|------|------|------|------|
| Brucellergen      | 11                            | 11 | 2    | 5    | 4    | 3    |
| Brucella Vaccine  | 7                             | 12 | 3    | 5    | 3    | 1    |

#### DISCUSSION

The high incidence (36 per cent) of positive intradermal reactions found in this study cannot, of course, be considered representative of the population as a whole, since only 50 persons were skin tested. However, it does serve to emphasize the point that healthy, active adults, who have never had symptoms of brucellosis, may have strongly positive skin tests. It does not seem presumptuous to state that several of these persons, if possessed with mild neurasthenic symptoms, would be regarded in certain circles as cases of chronic brucellosis. With the present enthusiasm for this disease, there is a tendency to forget that more than a positive skin test is necessary to make a diagnosis of active *Brucella* infection.

The agglutinin titers were consistently low. In one-half of the group none developed, and only a few were as high as 1:80. There was no apparent correlation between positive skin tests and agglutinin production, for a significant number developed agglutinins following negative skin tests, and the titers were as high as in those in whom the intradermal reaction was positive. From a comparative standpoint it can be said that the response to the two skin test antigens is almost identical.

From these results it would appear that titers above 1:80 can be regarded as due to contact with *Brucella* other than that of the skin test. Since only titers above 1:80 are usually regarded as indicative of active infection, the effect of the skin test can be disregarded entirely. Using a carefully prepared antigen and a standardized technique for reading the agglutination tests, we feel considerable confidence in this point of view.

As has been reported by others,<sup>6</sup> the titers obtained with the rapid slide test and with the macroscopic tube test were in close agreement. For many purposes the slide test, which is very simple to perform, would seem a satisfactory

diagnostic method. The possibility that our own tube antigen is relatively insensitive is precluded by the close agreement in agglutination titers obtained with the rapid slide method and with the stock antigen of the United States Department of Agriculture.

Several patients with low-grade fevers have reacted similarly to the "normal" persons described above. However, in two patients with acute febrile illnesses, both of whom had temperatures of above 40° C., agglutinin titers of 1:320 developed two weeks following skin tests. The intradermal reaction was positive in one and negative in the other. This excess rise in agglutinin titers, associated with fever, is well known and must be borne in mind in the interpretation of all agglutination tests.

The patients that present the greatest diagnostic problems are those with little or no fever, but with symptoms suggestive of chronic brucellosis. We can say with considerable assurance that in them the skin test will not influence the interpretation of the agglutination test. Where the blood culture is negative, and a careful correlation of the other laboratory procedures with the clinical picture is essential, it is of considerable practical aid to have such a criterion in dealing with patients who have been previously skin tested.

#### SUMMARY AND CONCLUSIONS

1. The agglutinin response of apparently healthy adults to the two commonly used types of *Brucella* skin-testing antigens has been studied, since this is a possible source of confusion in the clinical interpretation of the agglutination test. Commercial products, Lederle's *Brucella* vaccine and Huddleson's brucellergen, were used for the skin tests, to make the results of more practical value.

2. Positive intradermal reactions were present in 36 per cent of the 50 persons studied.

3. Approximately one-half of those skin tested failed to develop agglutinins, and in the others the titers were low, the maximum being 1:80.

4. There was little, if any, difference in response to the two antigens, and there was no correlation between positive skin tests and agglutinin production.

5. The practical significance of these results is discussed, and it is suggested that, except when high fevers are present, the agglutinin response to *Brucella* skin tests need not cause confusion in the interpretation of the agglutination tests.

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# OBSERVATIONS ON THE ELECTROCARDIOGRAM IN EPILEPSY AND COMPARISON WITH ELECTROCARDIOGRAM IN SEIZURES FOLLOWING CONVULSANT DRUG THERAPY\*

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THE introduction of convulsive drugs into the treatment of mental disease has stimulated interest in the study of the physiologic changes associated with the seizures resulting from the use of these drugs, and also in the study of changes associated with convulsive seizures in general.

Bellet, Freed, and Dwyer<sup>1</sup> noted the electrocardiographic alterations during 58 shock treatments on 40 different patients during the hypoglycemic shock treatment of schizophrenia. In two-thirds of the shock treatments noteworthy electrocardiographic changes were obtained. These consisted of depression of the S-T segments, diminution of the height of the T wave, prolongation of the Q-T interval, auricular fibrillation, extrasystoles, shifting pacemaker, sinus arrhythmia, and sinoauricular heart block.

Orenstein<sup>2</sup> observed 15 schizophrenic patients treated with metrazol convulsions. Electrocardiographic changes varying according to the severity of the convulsions were noted following the convulsions. After a mild seizure there were a rapid rate, T-wave elevation, depression of S-T segment, and arrhythmia. After a severe convulsion he found in addition, sinus arrest, shifting of the pacemaker, auricular and ventricular premature contractions, coupling of beats, nodal escape, and rarely a marked bradycardia.

Messinger and Moros<sup>3</sup> studied the electrocardiographic findings in patients before and immediately after metrazol convulsions. In no instance did they find any significant deflection of the S-T interval. In 3 of 10 cases they found a slightly diminished amplitude of the T waves, and in 7 others a slightly increased amplitude of the T waves.

Levine, Piltz, and Reznikoff<sup>4</sup> also studied the effects on the electrocardiogram of metrazol shock therapy and found no significant changes. In one patient they found inversion of the T wave in Leads II and III after the seizures. Since they believed this to be due to anoxemia, they gave the patient 100 per cent oxygen by inhalation for fifteen minutes. Thirty minutes later an electrocardiogram was taken; it recorded that most of the T waves in Leads II and III were upright.

\*From the Veterans Administration Facility, Minneapolis.

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Lennox and Cobb<sup>5</sup> obtained electrocardiographic records of patients during petit mal and myoclonic seizures, and in these instances neither the rhythm nor the amplitude of the waves was appreciably affected.

Erickson,<sup>6</sup> in a clinical study of changes in the circulatory system during or immediately preceding an attack of epilepsy, observed the electrocardiographic picture also. In most cases only one lead of the electrocardiogram was taken in order to avoid picking up too many electrical effects from the muscles during the convulsion. He used a direct chest lead, one electrode being placed over the manubrium sterni just below the sternal notch and the other just below the point at which the apex beat of the heart was palpated. He obtained satisfactory electrocardiographic tracings during 54 epileptic seizures of various types in 17 different patients. In none of the 54 attacks observed was there any dropping out of the QRS interval of the electrocardiogram. The T wave was unchanged in all but 4 patients. In 4 the T wave became biphasic or flattened at the end of the attacks.

Winternitz<sup>7</sup> observed a patient in status epilepticus, and for several days after the occurrence he noted changes in the electrocardiogram, a long R-T segment.

#### NEW DATA

The present study concerns mainly the electrocardiographic findings in a group of 20 epileptics observed at the Minnesota State Colony for Epileptics at Cambridge, Minn. Electrocardiograms were taken before seizures, during seizures, and at various intervals after seizures. Three standard leads and Lead IVF, as adopted by the American Heart Association, were taken on each patient. Thirty-eight electrocardiograms were taken in this series. The age of the patients varied from 13 years to 45 years. There was neither history nor clinical signs of heart disease in any of them.

Diminished amplitude of the T waves and increased depth of the T wave, where the T wave was normally inverted, were noted in practically every instance after the seizures. Other significant changes were observed in 5 of 12 patients in whom comparative electrocardiograms were made during seizures and in the period of remission. These changes consisted of variations of the electrical axis, changes in rhythm, various degrees of auriculoventricular block, dislocation of the pacemaker, and inversion of the T waves. Some of these changes were noted immediately after the seizures, and others for periods as long as four and one-half hours after a seizure. In one patient a tracing taken immediately after the termination of a convulsive seizure showed a negative T wave, whereas a positive T wave was noted immediately preceding the seizure. Another patient showed a change of electrical axis to right axis deviation, ventricular extrasystoles, and negative T waves in Leads I and IV after the seizure, and a normal electrocardiogram during the period of remission. A detailed description of the significant electrocardiographic findings is given in Table I.

A comparison of the electrocardiographic findings we observed following epileptic seizures, with those noted by other workers following convulsive seizures



induced by insulin shock and metrazol therapy of schizophrenia, reveals the fact that the changes are similar in character in each instance. This would lead us to believe that the physiologic changes preceding the convulsion and during the convulsive seizures are the same whether the convulsions are the result of insulin shock, metrazol, or epilepsy. Also in epileptic seizures there is an intrinsic factor which acts on the cerebral circulation and the cardiovascular circulation in general, in a manner similar to the extrinsic factors, insulin, and metrazol in their respective convulsive seizures.

TABLE I

| NAME   | AGE | SEX | TIME OF SEIZURE                   | DATE     | TIME OF EKG | INTER-VAL | EKG FINDINGS   |
|--------|-----|-----|-----------------------------------|----------|-------------|-----------|--|
| F. J.  | 15  | F   | 7 A.M. 8 A.M.                     | 12/11/39 | 1:15 P.M.   | 2 hr.     | T wave diminished in height  |
|        |     |     | 10:45 A.M.<br>None                | 1/ 8/40  | 10:25 A.M.  | 30 min.   | Negative   |
| M. Sw. | 38  | F   | 9 A.M.                            | 12/11/39 | 1:28 P.M.   | 4 hr.     | Slight T wave changes. Variations in height of R in Lead IV                                      |
|        |     |     | None                              | 1/ 8/40  | 1:45 P.M.   | 28 min.   | Negative   |
| G. U.  | 16  | F   | 8 A.M. 8:30 A.M.                  | 12/11/39 | 11:20 A.M.  | 2 hr.     | Ventricular extrasystoles; right axis deviation. Negative T wave in Leads I and IV               |
|        |     |     | None                              | 1/ 8/40  | 11:30 A.M.  | 50 min.   | Negative   |
| M. St. | 19  | F   | 6 A.M.-7:30 P.M.<br>(28 seizures) | 12/11/39 | 11:00 A.M.  | None      | Diminished height R and T waves. Wandering pacemaker. Negative T wave in Lead III during seizure |
|        |     |     |                                   |          | 1:50 P.M.   |           | Increased height of R and T waves  |
|        |     |     | None                              | 1/ 8/40  | 11:20 A.M.  |           | Negative   |
| L. F.  | 22  | F   | 6 A.M.-8 P.M.<br>(10 seizures)    | 12/11/39 | 11:40 A.M.  | None      | Diminished height R and T waves. Increased depth T wave during seizure                           |
|        |     |     |                                   | 1/ 8/40  | 11:10 A.M.  |           | Negative   |
| G. G.  | 21  | F   | 2:50 P.M.                         | 12/11/39 | 3:10 P.M.   | 20 min.   | T wave changes. Ventricular extrasystoles  |
|        |     |     | None                              | 1/ 8/40  | 1:50 P.M.   |           | Negative   |
| V. D.  | 45  | M   | 11:00 A.M.                        | 5/22/39  | 11:30 A.M.  | 30 min.   | Delayed auriculoventricular conduction with variable P-R interval (ventricular escape)           |
|        |     |     | None                              | 5/25/39  |             |           | Negative   |

## POSSIBLE CAUSES OF EKG CHANGES

Clinical observation and experimental research by many observers have shown that electrocardiographic changes similar to those observed following convulsive seizures are an indication of myocardial ischemia or anoxemia of the heart muscle.

Kountz and Gruber<sup>8</sup> produced anoxemia in animals, and when the oxygen saturation fell below 50 per cent, electrocardiographic changes similar to those observed clinically in coronary occlusion were found. Release of the animals from the anoxic state caused the electrocardiogram to return to normal. They

also produced pictures similar to anoxemia by the injection of pitressin, the primary action of which is vasoconstriction. The electrocardiographic changes in these instances could be made to disappear by the use of vasodilators, such as sodium nitrite. Milles and Smith<sup>2</sup> produced similar changes by the intravenous injection of epinephrine. It may be assumed, therefore, that the electrocardiographic changes seen following convulsive seizures from insulin shock, metrazol, and epilepsy are due to myocardial ischemia or anoxemia of the heart muscle.

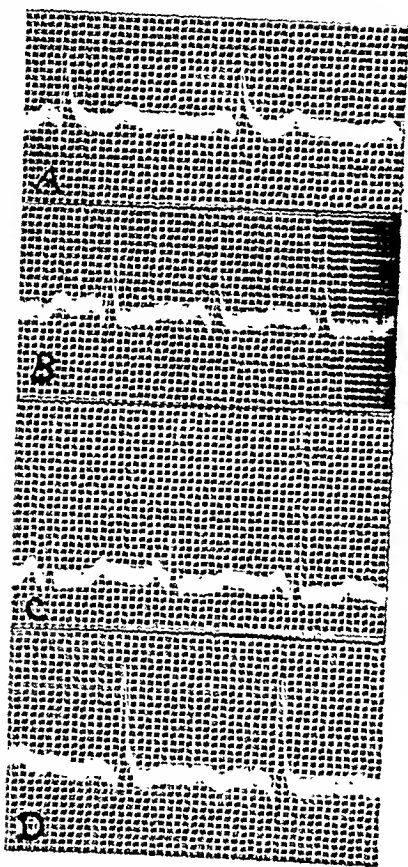


Fig. 1.

Fig. 1.—EKG Lead III showing T-wave changes after seizures. A, (12/11/39) 11:00 A.M.—one-half hour after seizure. B, 11:20 A.M. During seizure. C, 1:50 P.M. two and one-half hours after seizure. D, Control (1/8/40). No seizures.

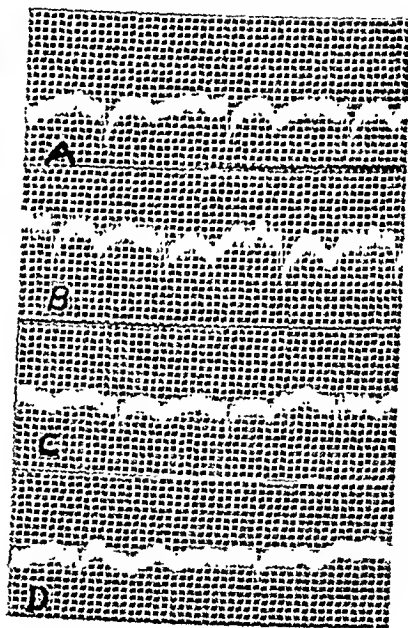


Fig. 2.

Fig. 2.—EKG Lead III showing T-wave changes after seizures (12/11/39). A, 11:40 A.M.—one hour after seizure. B, 11:45 A.M. During seizure. C, 1:45 P.M.—two hours after seizure. D, Control (1/8/40). No seizures.

Bellet, Freed, and Dwyer<sup>3</sup> believe that in insulin shock therapy the lack of available carbohydrates and the increased work of the heart during hypoglycemic shock of long duration suggest that anoxemia may be a factor in producing the cardiac effects. They base their conclusions on previous investigations which have shown that lowering of the blood sugar reduces the oxygen consumption of the brain; also that the profound changes which occur in cerebral metabolism during hypoglycemia and the resultant coma may indirectly affect the heart.

Messinger and Moros<sup>3</sup> formulate an hypothesis to account for the diverse cardiovascular changes they noted in their study of the effects of insulin and metrazol in these cases. They are of the opinion that persons differ in their response to various types of stimuli, and particularly in the degree of their response to drugs of the sympathetic-adrenal type. It is now recognized that the prolonged administration of insulin (as in shock treatment) may induce an altered state of reactivity of the sympathetic system primarily in the direction of an increased sensitivity of response to adrenalin and sympathetic-mimetic

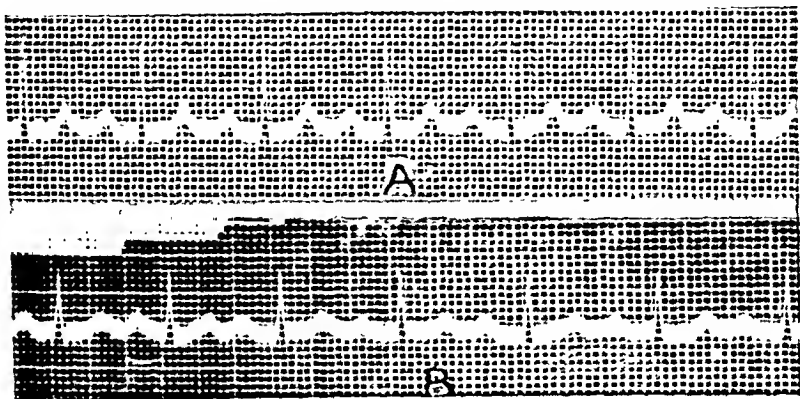


Fig. 3.—EKG showing delayed auriculo-ventricular conduction with variable P-R interval. A, Control (5/25/39). Normal tracing. B, Thirty minutes after seizure (5/22/39) showing delayed auriculoventricular conduction with variable P-R interval, simulating ventricular escape.

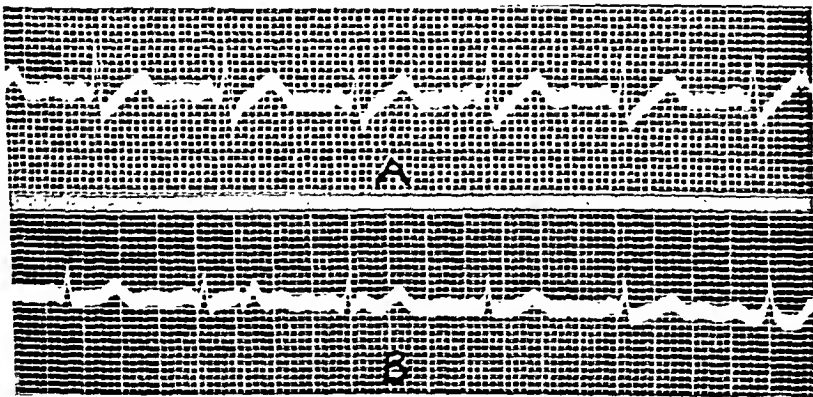


Fig. 4.—EKG showing wandering pacemaker after a seizure. A, Control (1/8/40). Normal tracing. B, After several seizures (12/11/39) showing variable P waves, indicating a wandering pacemaker.

drugs. Also metrazol may produce transitory effects which are typical of a mild hyperadrenalemia. That adrenalin may cause heart changes has been shown by the work of Milles and Smith<sup>9</sup> and others. These observers showed that electrocardiographic changes similar to those occurring in convulsive seizures could be produced by the intravenous injection of adrenalin.

Levine, Piltz, and Reznikoff,<sup>4</sup> contending that anoxemia might account for the electrocardiographic changes seen after metrazol therapy, gave their patient, who showed an inverted T<sub>2</sub> and T<sub>3</sub> after metrazol convulsions, 100 per cent

oxygen by inhalation for fifteen minutes. Thirty minutes later the electrocardiogram revealed that most of the T waves in Leads II and III were upright.

In the study of chemical and physiologic conditions, which may be contributing factors to epileptic seizures, four major conditions are usually given; namely, acid-base balance, fluid balance, oxygenation, and circulation. That these factors may be interrelated and responsible for cardiovascular changes in epilepsy, as depicted by the electrocardiogram, may be assumed from our observations on this group of patients which we studied.

Reduction of alkalinity and induction of acidosis, either by fasting, physical exercise, or the ketogenic diet, is known to precipitate seizures.

McBroom,<sup>10</sup> in collaboration with Hirschfelder and Haury,<sup>11</sup> made a study of blood potassium in epilepsy. They found a gradual increase in the potassium up to the time of seizure, followed by a drop immediately after the seizure.

Moglia<sup>12</sup> studied the effects of adrenalin on the potassium in the plasma. He injected adrenalin intravenously into chloralosed dogs at a constant rate for fifteen minutes. A dose of 5 gamma per kilogram per minute produced an initial increase of plasma potassium, lasting from five to ten minutes, followed by a prolonged decrease. A dose of 0.5 and 0.25 gamma produced a prolonged decrease without the initial increase. The larger doses were similar to the amounts secreted by the adrenals in states of emergency; the smaller ones were similar to the physiologic amounts of adrenalin and sympathin.

Thomson<sup>13</sup> studied the effect of potassium on the heart by giving potassium chloride and citrate in doses varying from 3 to 30 Gm. daily to 24 patients with various diseases, and observed the effects on the electrocardiogram. In 14 patients there was an increased height in the T waves in one or more leads after giving the potassium. The average increase in the level of the serum potassium was 8.3 mg. per 100 c.c., as compared with 2.6 mg. per 100 c.c. in the patients who showed no such change. In one patient the T wave became more diphasic. In two patients definite degrees of heart block were produced. In one there was prolongation of the P-R interval. Other changes also were noted. Thomson advances the hypothesis that these changes may be mediated through the action of the potassium ion on the myocardium because of the similarity of these changes to those produced by vagal stimulation, by acetylcholine, and by pitresin.

Changes in the cerebral circulation and also in the general circulation, probably resulting from this vasoconstrictor effect, have been shown by many observers. Penfield<sup>14</sup> reported the cessation of cerebral pulsations during convulsions occurring when the brain was exposed by craniotomy.

Olkon<sup>15</sup> studied the microscopic vascular changes in the brain, internal viscera, and skin of living animals in relation to convulsions. Following convulsions induced by the injection of excitant drugs, capillary changes were invariably found in the pia-arachnoid, skin, and internal organs. These consisted of twisting, irregularity, and deformity, and a sluggishness in the circulating cells within their lumen.

Denyssen and Watterson<sup>19</sup> also are of the opinion that a vasoconstrictor effect is responsible for the convulsions. They gave vasodepressor drugs, such as amyl nitrite, sodium nitrite, and histamin, prior to the administration of convulsive doses of metrazol. No fits occurred.

### CONCLUSIONS

The electrocardiographic findings which we have observed during and after epileptic seizures tend to show that cardiovascular changes occur following epileptic seizures, and that these changes are part of circulatory changes throughout the body. As a result of this, and from the experimental and clinical observations of other workers, the supposition is made that the epileptic seizures are due to a cerebral anoxemia resulting from an ischemia of the brain following vasoconstriction. This is probably the result of an electrochemical change and adrenalin-like action on the cerebral vessels.

After comparing our findings with those of other workers who studied the electrocardiographic changes following seizures after convulsant drug therapy, we conclude that the physiologic changes during and after convulsive seizures from excitant drugs are the same as those due to epilepsy; also that the extrinsic factors, such as insulin shock and metrazol, probably produce a similar adrenalin-like reaction as the intrinsic factor which brings on the cerebral ischemia and anoxemia in epileptic seizures.

We wish to thank Dr. D. E. McBroom, Superintendent, Minnesota Colony for Epileptics, and his associates for the opportunity to observe the patients at the institution and for their valuable cooperation and assistance in making this study possible.

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## PERIPHERAL NERVES IN CHRONIC ATROPHIC ARTHRITIS\*

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THE obvious fact that chronic atrophic arthritis is not solely a joint disease, but one in which many systems of the body are involved, has caused us to undertake a more detailed investigation of the entire organism. We have begun with a re-examination of all the pathologic changes. Recently, the muscles and valves of the heart have been examined and noteworthy findings have been reported. Because of the nature of the pains, trophic conditions, vasomotor alterations, and heightened reflexes, a more detailed study of the peripheral nerves and sympathetic chains were undertaken. In earlier studies only the brains and spinal cords were examined; no specific or characteristic lesions were found.



Fig. 1.—A longitudinally cut section through a femoral nerve of a patient with severe chronic atrophic arthritis,  $\times 9$ . The arrows indicate the perineural or interfascicular location of the nodules.

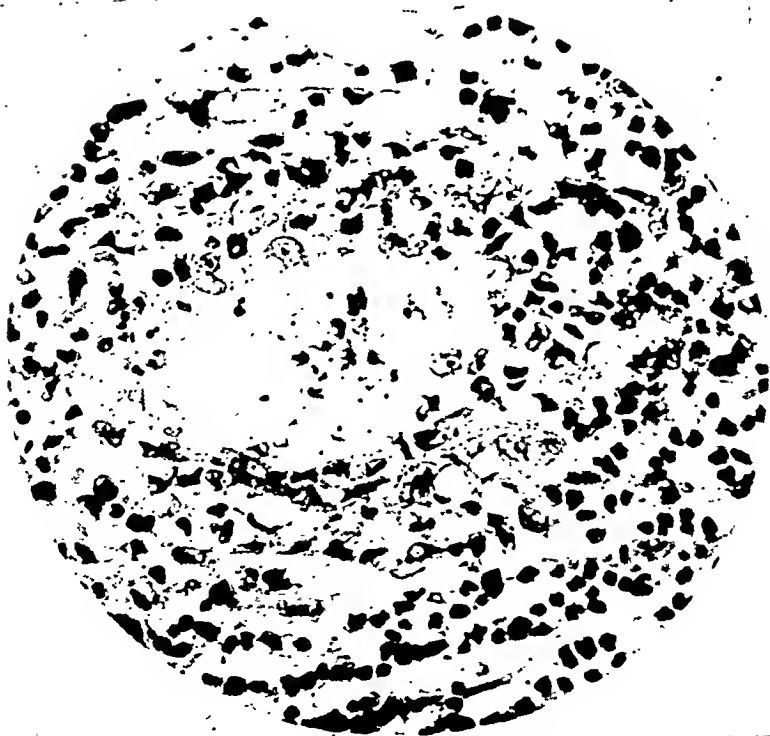
In our present study, covering the past two and one-half years, hitherto undescribed pathologic changes involving the peripheral nervous system have been found. These are recorded here briefly; a more detailed report will follow.

The brains of 11 patients with definite chronic atrophic (rheumatoid) arthritis and the peripheral nerves in five of the patients were examined. There were no specific changes in the central nervous system; however, in two of five cases of chronic atrophic arthritis characteristic and severe pathologic lesions

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A.



B.

Fig. 2.—A and B represent the same nodule in two different magnifications,  $\times 150$  and  $\times 550$ , respectively. Three distinct zones, the inner necrotic zone, the intermediate zone of proliferating mesenchymal cells, and the peripheral lymphocytic zone, are clearly shown. The intermediate zone in this nodule is somewhat smaller than usual.

were found in peripheral nerve trunks (sciatic, femoral, brachial plexus). In one case the lesion in the peripheral nerves was somewhat milder, and in the last two cases the changes were only slight. The characteristic findings were represented by sharply circumscribed nodules of round or oval shape. The nodules are not seen grossly in unstained specimens. However, they can be seen with the naked eye in stained slides. Fig. 1 represents a longitudinally cut part of a peripheral nerve magnified nine times to show the distribution of the nodules in a peripheral nerve. The nodules are located in the perineurium and are less common in the epineurium; they are never found in the endoneurium of the peripheral nerves. The size of a single nodule varies. Serial sections through a single nodule show an involvement 0.14 to 0.2 mm. in diameter. The nodules are often found in groups of three or four in the same longitudinally cut section. Histologically, each nodule consists of two or three zones: a central zone of necrosis which may or may not be present, an intermediate zone of proliferating mesenchymal cells, and a peripheral ringlike zone of inflammation with lymphocytes and plasma cells. The cells in the intermediate zone are of the so-called epithelioid type. They are polyhedral and have large elongated irregular nuclei, poor in chromatin. Clear cell outlines are usually not demonstrable. Fig. 2 *A* and *B* are photomicrographs,  $\times 175$  and  $\times 550$ , respectively. The three zones of the nodule are clearly seen. Conglomeration into larger areas is never found. This finding has to be classified as "rheumathritic nodulous perineuritis." The specificity of this change has been confirmed by examination of peripheral nerves from 86 control cases which were examined without knowledge of the clinical history or the findings at autopsy. Six cases showed pathologic changes in the peripheral nerves. In one case with the finding of perineuritic nodules the clinical history revealed that the patient had chronic atrophic arthritis for many years. Recently in continuing our control examination, a new case with perineuritic nodules in peripheral nerves was found. This was an 80-year-old female with old rheumatic heart disease (clinically diagnosed as rheumatoid arthritis). The pathologic changes in the five nonarthritic control cases (one of Buerger's disease, two of dermatomyositis, one of postencephalitic parkinsonism, one of carcinomatous infiltrations of the epineurium) differed distinctly from the perineuritic changes in chronic atrophic arthritis. We consider rheumathritic nodulous perineuritis a specific active inflammatory process concomitant and parallel with other rheumatic pathologic features in chronic atrophic arthritis, such as rheumatic heart disease, subcutaneous nodules, and synovial inflammation and proliferation.



## BRUCELLOSIS: STUDIES EMPHASIZING STRAIN VARIATION IN SEROLOGIC TESTING\*

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THE purpose of this study is to explain the wide variation in agglutination titer and opsonocytophagic activity which we have observed from various laboratories. In many cases the difference in results were too great to be accounted for as technical variations. To illustrate, a state laboratory reported a negative *Brucella* agglutination titer on the same serum which we were able to demonstrate a positive titer of 1:640 with three commercial antigens. In another instance, the laboratory using a single strain of the *Brucella* reported 69 per cent of polymorphonuclear neutrophils showing marked phagocytosis. One week later we did not find marked phagocytosis in any of the cells. Therefore, we outlined a plan to investigate the agglutination titer and cytophagic activity when the tests were performed by the same technician with 20 different strains.

In this study we selected 34 patients from private practice. From all, blood serum had yielded positive agglutination tests either in our laboratory or in other laboratories. The antigens were prepared from 20 smooth strains of the *Brucella* obtained from the universities of Michigan, Minnesota, Illinois, Wisconsin, and the Public Health Laboratory in Washington. All strains were negative to thermoagglutination and dye agglutination before and after the experiment. All strains were injected into guinea pigs. Fourteen strains produced death, 8 of which showed gross *Brucella* lesions and 6 of which produced microscopic changes only. The other 6 showed evidence of disease by loss of weight, roughening of hair, comparatively low white blood cell counts and maintenance of high agglutinin titers. All strains were determined to react with known positive and negative bloods.

*Opsonocytophagic Tests.*—Suspensions were prepared from forty-eight-hour cultures of *Brucella* organisms in saline with pH 7.02 to 7.1. Turbidity was adjusted to McFarland nephelometer No. 25. Five cubic centimeters of blood were collected in sterile tubes that had been filled with the proper amount of citrate to give a final concentration of 0.8 per cent and marked to the level to which the blood should be added. All tests were completed within four hours after the blood was collected. Performance of the test was that described by Huddleson,<sup>1</sup> except that smears were made by the cover slip method since this method, in our opinion, is superior to the slide method in that less mechanical injury to the cells is induced when blood spreads by capillarity rather than by

\*From the Hixon Laboratory for Medical Research, University of Kansas, by the Boylan Foundation.

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actual smearing. Smears were stained with Hasting's stain. One hundred cells were counted; the findings are, therefore, expressed as percentage of cells which showed marked, moderate, weak, and negative cytophagic activity.

**Agglutination Test.**—Strain antigens were prepared by washing a seventy-two-hour culture of *Brucella* from liver infusion slants with saline containing 0.5 per cent phenol. The suspensions were strained, centrifuged, and resuspended. The stock antigens were stored as concentrated suspensions. The dilution was adjusted to McFarland nephelometer No. 1.5 for each antigen, recorded, and made up as needed. Twenty cubic centimeters of patient's blood were collected in a chemically clean tube, rimmed, and centrifuged. The serum was removed and stored at 7° C. until the agglutination series could be run within the next two days.

| Tube     | 1       | 2     | 3     | 4     | 5     | 6     | etc. |
|----------|---------|-------|-------|-------|-------|-------|------|
| Antigen  | 1.8 cc. | 1 cc. | 1 cc. | 1 cc. | 1 cc. | 1 cc. | etc. |
| Serum    | .2 cc.  | 1 cc. | 1 cc. | 1 cc. | 1 cc. | 1 cc. | etc. |
| Dilution | 1:10    | 1:20  | 1:40  | 1:80  | 1:160 | 1:320 | etc. |

Fig. 1.

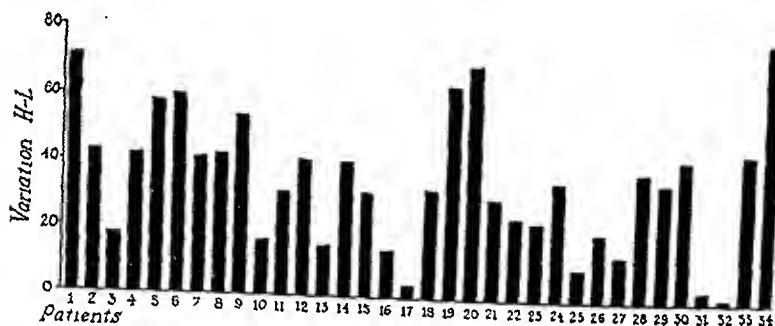


Chart 1.—Range of variation of opsonocytophagic index numbers, as determined by Foshay-LeBlanc method, in 34 patients with brucellosis when tested with 20 strains of *Brucella*.

To prevent numerous pipettings, the agglutination test was performed as shown in Fig. 1. The method is more accurate by reason of fewer operations in pipetting small amounts, and is a timesaver as well. The tests were incubated at 37° C. in a hot-air incubator for twelve hours and allowed to stand at refrigerator temperatures for twenty-four hours, after which readings were made. The titer was read from the last serum dilution showing complete agglutination, that is, the supernatant fluid completely clear with the bacteria completely sedimented in clumps.

#### EXPERIMENTAL RESULTS

Charts 1 and 2 record our results. Chart 1 represents the correlation of results of the opsonocytophagic tests calculated by the Foshay-LeBlanc method,<sup>2</sup> the final results being obtained by subtracting the minimum index from the maximum index to determine the variation when each serum was set up against 20 strains of *Brucella* simultaneously.

Chart 2 shows the correlation of agglutination titers demonstrating the variation in agglutinin by subtracting the minimum titer from the maximum titer in terms of number of tubes apart in the test set-up.

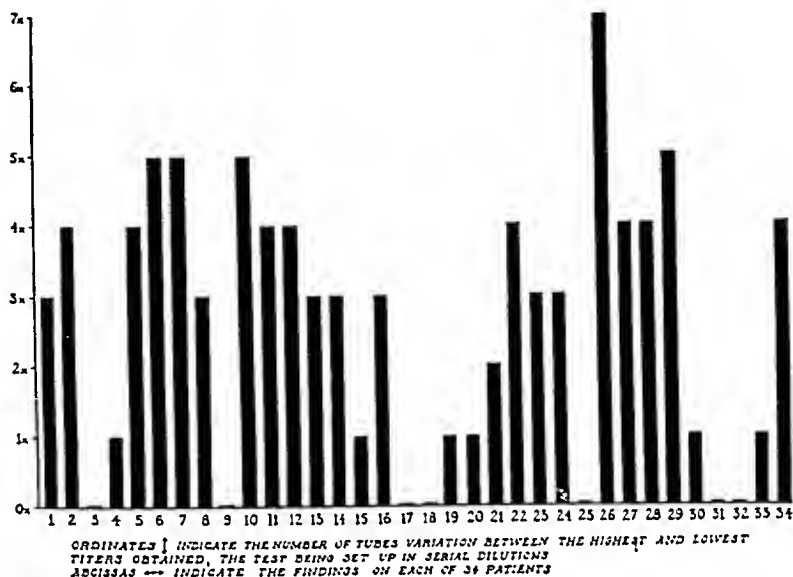


Chart 2.—Variation in agglutinin titers of patients with brucellosis tested with antigens from 20 strains of Brucella.

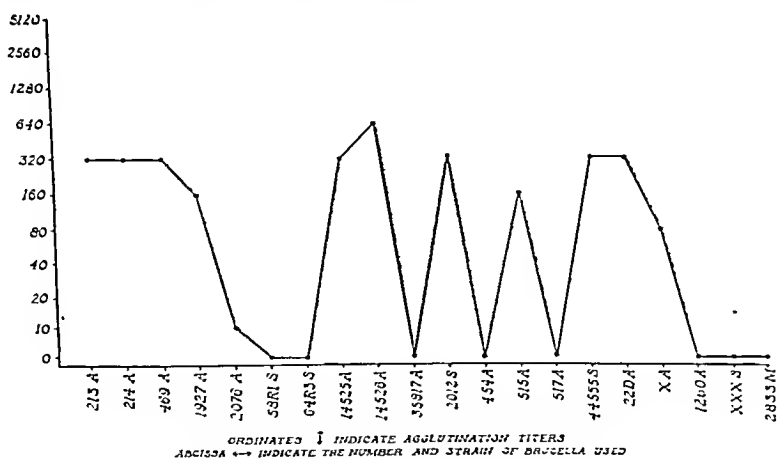


Chart 3.—Agglutination series on a single patient (EM-26), showing marked variation of agglutination titers when tested with 20 strains of Brucella.

It is observed from the studies that there is wide fluctuation in the majority of instances. When there was little variation, as in the patients 17, 31, 32 in Chart 1, their actual opsonocytophagic activity was almost negative; similarly, when the records of patients 3, 9, 17, 18, 25, 31, 32 in Chart 2 are examined, their agglutination titers were negative throughout. When there was only a difference of one tube, probably not outside the range of individual variation, the titers were either very low (1:10) or very high (1:2,560 to 1:5,120 and 1:1,280 to 1:2,560). In the analysis of Chart 2 a variation is noted in the study of the

agglutination reaction, the widest being from no agglutination to complete agglutination in a dilution of 640 in the case of EM-26. This is shown graphically in Chart 3. The range of agglutination is from 0 to 1:640. Ten strains were agglutinated in a dilution of 1:160 or above. Eight strains were not agglutinated.

#### DISCUSSION

It will be noted from the foregoing results that there is wide variation between the agglutination and opsonocytophagic activity of the same blood taken at the same time and tested under the same conditions with the 20 strains employed.

A like observation was made in 1937 by Campbell and Greenfield,<sup>3</sup> employing four strains of *Brucella* in agglutination tests on patients with pyrexia in whom typhoid, paratyphoid, influenza, pyogenic and tubercular infections had been excluded. They found in one case agglutination tests varied from no complete agglutination with a *melitensis* strain to 1:1,600 complete with one *abortus* strain and 1:100 with another. The organism isolated from the patient's blood was *Brucella abortus*. Previously, in 1932, Plastring and McAlpine,<sup>4</sup> who employed agglutination absorption technique on 116 strains of *abortus*, indicated close agreement of antigenicity of strains, 85 per cent of which completely absorbed agglutinins from antisera of bovine and porcine types and from one *melitensis* antiserum. In their own words, "No serologic differences were observed between bovine and porcine types of *Brucella abortus*, regardless of origin." Fensier and Meyer,<sup>5</sup> as early as 1920, concluded, "Strains within the same group do not necessarily act in a uniform manner when absorbed from the same antiserum. This constitutes the basis for individual differentiation. In several *melitensis* strains, for example, the range of variability was so great that certain strains appeared to have little in common." Giordano,<sup>6</sup> Keefer,<sup>7</sup> Carpenter, Boak, and Chapman,<sup>8</sup> and Foshay<sup>9</sup> mention specifically that in routine testing variations in agglutination titers of patients were marked when separate strains were used to check. Agglutination absorption test and standard agglutination test apparently are not comparable, since from our results with *abortus*, *suis*, and *melitensis* strains we concluded that infecting *Brucella* strains must vary tremendously in antigenicity.

Huddleson<sup>1</sup> states that in routine testing an antigen containing one smooth strain of *abortus* is sufficient. To determine the smoothness of the strain, the method of choice is the phagocytosis of the strain with known negative and positive bloods as an index. Our strains were neither thermoagglutinable nor dye agglutinable and yet variation in phagocytic system was marked. We feel that variation in the phagocytic system was a strain difference, not necessarily associated with its smoothness or roughness.

#### CONCLUSIONS

1. A series of 34 patients diagnosed as having chronic undulant fever has been examined, employing agglutination and opsonocytophagic tests.

2. Marked variation in both agglutinin titers and opsonocytophagic indices has been found. This indicates the necessity for standardization of *Brucella* polyvalent antigens for agglutination tests.

3. Testing with five to ten smooth strains for cytophagic activity is necessary to obtain reliable results.

4. From our studies it would appear that the diagnosis of acute or chronic brucellosis is dependent upon clinical opinion with some positive immunologic test.

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## RECOVERY OF RABIES VIRUS FROM THE BRAIN OF AN UNDIAGNOSED CASE\*

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THE symptoms of rabies are usually so typical that when accompanied by a history of a dog bite, the case will appear to follow the classical description closely, and the diagnosis is rarely missed. When, however, a history of a bite is not previously established, and a case presents itself during a late stage of the disease, a correct diagnosis may then become exceedingly difficult. It is possible, therefore, that an occasional death which follows a symptomatology obscured by the lack of an incriminating history of contact with a rabid animal, may actually be the result of infection with rabies virus.

The belief has been expressed that this may occur much more frequently than may be supposed.<sup>1-3</sup> An excellent illustration of such an occurrence is described in the following report, which deals with the isolation and identification of rabies virus from a case that came to the Medical Examiner for autopsy because the symptomatology and cause of death were obscure.

In the afternoon of Feb. 15, 1940, S. G., white, 49 years old, residing on the lower East Side of New York, walked into the Columbus Hospital and asked to be admitted for observation. He complained of difficulty in swallowing, which he stated was "because of poison in

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\*From the Bureau of Laboratories, Department of Health, New York City.  
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throat placed there by someone." After admission the patient became unmanageable, wildly delirious, and induced emesis which resulted in the vomiting of black fluid mixed with blood. The diagnosis was "psychoneurosis" and "acute state of anxiety." He was about to be transferred to the psychopathic ward at Bellevue Hospital, when he died at 10:00 A.M. on February 16. The body was submitted to the Medical Examiner, and a post-mortem examination was made by Dr. Eugene Clark on February 17.

There was no evidence of injury, except for abrasions on the dorsum of the hands, on the middle finger of the right hand, and a healing wound, irregularly shaped and  $1\frac{1}{2}$  inches long, on the right index finger. The meninges were normal. There was a slight subarachnoid hemorrhage at the posterior pole of the cerebrum. No macroscopic lesions were found in the pons, medulla, or elsewhere in the brain. Aside from a general visceral congestion, no special pathology could be observed. Opinion as to the cause of death was, therefore, reserved, pending chemical and microscopic examination.

On the day of autopsy portions of the brain in glycerin and in 10 per cent formalin were submitted to us for investigation of a possible central nervous system virus infection.

For aid in the pathologic examination of the fixed tissues, we secured the assistance of Dr. Vera B. Dolgopel, of the Willard Parker Hospital. Her opinion was: "Sections from medulla show a number of veins with several rows of lymphocytes within the walls. The perivascular space is free from exudate, but is markedly dilated. Some of the veins with cellular exudate are situated near the ventricular surface, while others are present on the lateral aspect of the medulla and near the olives. No glial nodules are observed. The ganglion cells show no remarkable degeneration. The section from the brain shows marked congestion of veins but no vascular infiltration. Microscopic diagnosis: bulbar myelitis."

Because it was inconvenient to do animal inoculations at the time the glycerinated brain material was received, it was washed several times in saline solution to remove the glycerin, then frozen and stored in a dry-ice cabinet at  $-76^{\circ}$  C. for forty-eight hours. The portions of brain were then thawed rapidly in the incubator at  $37^{\circ}$  C., triturated in a mortar to a fine pastelike consistency, and suspended in broth to a concentration of about 20 per cent. The suspension was centrifuged at about 2,000 r.p.m. for ten minutes, and the supernatant fluid was inoculated intracerebrally into animals. Each of six mice received 0.03 c.c., each of two guinea pigs 0.25 c.c., and each of two rabbits 0.5 c.c. Cultures in broth were made simultaneously to determine the bacterial content of the suspension. The results of these inoculations and all successive passages made thereafter are indicated in Fig. 1.

All material for subsequent inoculations consisted of a 10 per cent suspension of a pool of several mouse brains or, as in the one case indicated, of a single guinea pig brain. The volumes inoculated throughout were the same as those stated above.

The two inoculated guinea pigs were found dead the following morning. Smears and cultures from the brains of these animals showed heavy bacterial growth, with predominance of a gram-positive bacillus and fewer gram-positive cocci. These were also present in cultures of the original brain material.

Fortunately, the mice resisted these contaminants, and on the ninth day after inoculation two mice became inactive and showed a weakness of the hind legs. Several hours later they became moribund and were thereupon promptly killed. Their brains were removed with aseptic precautions, a portion of each was inoculated into culture media and the remainder was frozen at  $-76^{\circ}$  C. As

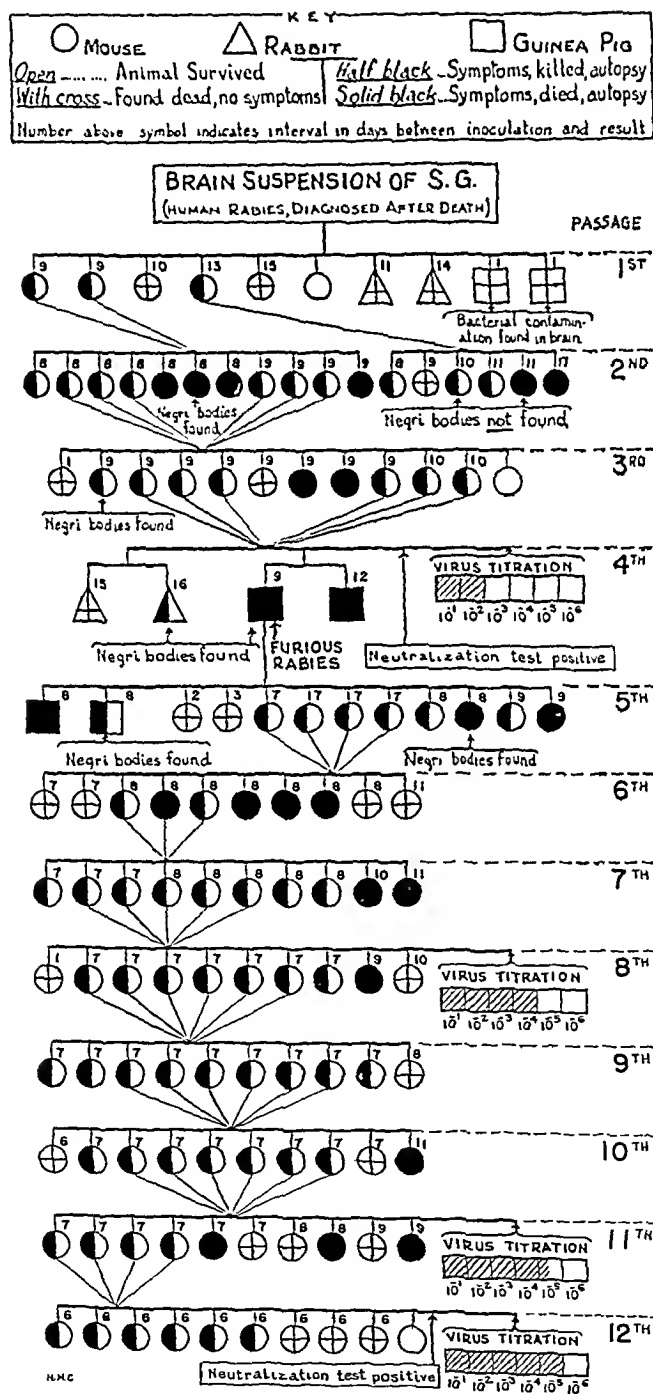


Fig. 1.—Animal passages and tests with virus isolated from a case of human rabies.

no growth was observed in the cultures, a 10 per cent suspension of these brains was prepared and inoculated into a group of ten mice. On the thirteenth day a third mouse of the originally injected group of six showed symptoms similar to those observed in the first two, and a suspension of its brain proving bacteriologically sterile was inoculated into a group of six mice.

The two rabbits were found dead in their cages on the eleventh and fourteenth day, respectively. Bacteriologic examination of the brains of both these animals yielded negative results, and they were stored at  $-76^{\circ}\text{C}$ ., pending the outcome of the mouse passages.

Beginning with the second passage, the mice began to show more definite symptoms, usually starting with paresis, rapidly developing paralysis, and terminating in prostration and death. Since serial passage of the brains of animals thus affected resulted in a clear-cut, reproducible clinical syndrome, we inferred that an infectious agent, probably a neurotropic virus, was involved.

Judging from the case history, incubation period, and clinical symptoms in mice, the viruses first to be considered logically appeared to be those of rabies, herpes, or encephalitis.

Accordingly, the brain of one mouse in the second passage was examined for Negri bodies by direct smear, and two brains were sectioned for histopathologic study. No Negri bodies were found on direct smear. The histologic sections showed mononuclear cuffing around small veins in the subcortical structures and the cortex. There was no diffuse meningitis, although occasionally groups of lymphocytes were present in limited areas of the meninges. A large number of intracellular and extracellular Negri bodies were seen in one of the sections of the hippocampus.

In subsequent passages, namely, the third, fourth, and fifth, Negri bodies were easily demonstrated by direct smear. These were seen also in the brains of a rabbit and a guinea pig\* inoculated with third passage mouse brain and in the brain of another guinea pig receiving a further guinea pig brain passage. None of the brains obtained in the remainder of passages was examined for Negri bodies.

The finding of Negri bodies made it fairly obvious that rabies virus had been isolated. It was nevertheless deemed necessary to conduct neutralization tests in order to demonstrate the serologic specificity of this virus. The virus was, therefore, tested against an antirabies serum prepared by the injection of rabbits with a Department of Health strain of fixed rabies virus. The serum definitely neutralized the isolated virus in this test, but because of the low infectivity of the virus in its third passage, further passages were resorted to so that the potency of the virus might increase sufficiently and a repetition of the neutralization test could be made under more suitable conditions.

The chart of the virus passages shows quite clearly that a gradual rise in the infectivity titer took place, starting at  $10^{-2}$  in the third passage to  $10^{-5}$  in the eleventh passage. It will also be noted that correspondingly there was a decrease in the incubation period from nine days or more at the beginning to a uniform six-day incubation period in the last passage made.

\*This animal exhibited symptoms characteristic of furious rabies. It became excitable, ran wildly about in its cage, frothed at the mouth, and repeatedly attacked and bit its cage mate. Finally, after repeated convulsive seizures, it became prostrate and died.



A virus preparation obtained from a pool of eleventh passage mouse brains was again tested against the antirabic serum. The results of the second test fully confirmed the findings of those in the first in a clear-cut and satisfactory manner.

As soon as sufficient evidence was obtained to be certain that we had uncovered a case of undiagnosed rabies, further investigation was made by the Bureau of Preventable Diseases, and the following facts were elicited:

From an interview with the son of S. G. it was learned that S. G., while at play with his dog on December 25, was bitten on the index finger of the right hand. He applied tincture of iodine and a bandage to the bleeding wound, but sought no medical advice. The son, P. G., was bitten on the left leg on the same day. He also applied tincture of iodine with no further treatment. The dog subsequently vanished and was not seen thereafter, but P. G. added the further information that the dog had been ill for two days prior to December 25 with vomiting and "fits," and had tried to bite people on the street. He also stated that the dog showed scars on its face as a result of having been bitten by another dog two weeks previously.

S. G. felt well and worked as a laborer until February 13 when he developed fever. On February 14 he could not drink or swallow, had dryness of the mouth, and feared the sight of water. A local physician examining him that day diagnosed his condition as that of grippe.

On February 15 a Hospital ambulance interne refused to remove him to the hospital, stating he was not sick enough for hospitalization. The same day S. G. went to Columbus Hospital where he was admitted. Death ensued the following day.

This information, furnished by the Bureau of Preventable Diseases, served to complete the history of a perfectly typical case of rabies which, baffling as it appeared in the beginning, might have remained undetermined and unrecorded were it not for the first clues obtained in the laboratory.

To the physician and public health officer this report may be of significance, since it indicates the need to be more watchful for cases of unsuspected rabies in areas where this disease is endemic.

#### SUMMARY

With the utilization of laboratory procedures, rabies virus was recovered from a person, the cause of whose death was obscure. The presence of virus in the brain of this undiagnosed case was established by animal passages, by the demonstration of Negri bodies in the brains of inoculated animals, and by immunologic methods.

Following the recovery of virus, further investigation revealed that the history of a dog bite and other data pertinent to a typical case of rabies also existed.

The need to be ever mindful that such cases may occur in communities where rabies is endemic is indicated by this report.

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# SULFONAMIDE THERAPY OF STREPTOCOCCUS INFECTIONS BY THE INTRAVENOUS DRIP METHOD\*

WITH A NOTE ON THE TOXICITY OF NEOPRONTOSIL BY INTRAVENOUS ADMINISTRATION

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WITH THE TECHNICAL ASSISTANCE OF ANNA M. RULE AND LORAINÉ GROSKIN

THE massive arsenotherapy of early syphilis by the intravenous drip injection of solutions of neoarsphenamine or mapharsen over a period of five days, proposed by Chargin, Leifer, and Hyman,<sup>1, 2</sup> has suggested that this method of administration of the soluble sulfonamide compounds may be useful in the treatment of streptococcal and other septicemias.

However, since therapeutically effective concentrations of free sulfonamide compounds in the blood may be maintained in the majority of persons by oral or parenteral administration of adequate doses at suitable intervals, the indications for their administration by the continuous intravenous drip method are not apparent, although worthy of investigation in relation to the chemotherapy of severe septicemia with special reference to the treatment of subacute bacterial endocarditis due to infection with *Streptococcus viridans*.

In this connection it may be stated that the massive arsenotherapy of acute testicular syphilis of rabbits by Kolmer and Rule,<sup>3</sup> consisting of the intravenous drip method of administration of neoarsphenamine and mapharsen once a day for five days in succession, did not prove any more effective than the daily intravenous injection of single doses by syringe for five days in succession, or single intravenous injections of the compounds, as the *total* minimal curative doses in relation to biologic cure were practically the same by all three methods of administration, being approximately 0.020 Gm. of neoarsphenamine or 0.005 Gm. of mapharsen per kilogram of weight.

## EXPERIMENTAL

Rabbits were chosen for the experimental part of this investigation because of their susceptibility to infection with virulent beta hemolytic streptococci of group A by intradermal or intravenous inoculation<sup>4</sup> and the ease with which solutions of sulfanilamide and neoprontosil could be injected intravenously by the drip method at daily intervals.

\*From the Research Institute of Cutaneous Medicine, Philadelphia.  
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On the other hand, it was realized that rabbits are not as suitable as mice for chemotherapeutic investigations in experimental streptococcus infections with the sulfonamide compounds largely because sulfanilamide and other sulfonamide compounds are more rapidly acetylated into compounds of reduced or doubtful therapeutic properties.<sup>5, 6</sup> Thus Kolmer, Brown, and Rule<sup>7</sup> have observed that about 83 per cent of untreated rabbits inoculated intradermally and intravenously at the same time with virulent beta hemolytic streptococci developed suppurative arthritis, which progressed to a fatal outcome with positive blood and joint cultures. In a series treated with sulfanilamide by intravenous administration about 75 per cent developed suppurative arthritis with positive blood and joint cultures, all of which terminated fatally. Better results, however, were observed in the treatment of rabbits with neoprontosil inoculated only intradermally, which produces a severe local lesion without an associated septicemia.<sup>5</sup>

The strain of beta hemolytic streptococcus (group A) employed in this investigation was of such virulence that the intravenous injection of adult rabbits, weighing from 2,000 to 2,800 Gm., with 0.8 c.c. of 18-hour broth culture, regularly produced infections fatal within fourteen days with the development of suppurative arthritis with positive joint cultures in about 75 per cent of animals. Heart blood cultures were usually positive at daily intervals during the first four or five days following inoculation after which they were usually sterile.

TABLE I

RESULTS OF TREATMENT OF RABBITS WITH SULFANILAMIDE AND NEOPRONTOSIL ADMINISTERED BY THE INTRAVENOUS DRIP METHOD\*

| COMPOUNDS          | NUMBER RABBITS† | DOSE PER KG. (GM.) | SURVIVALS: DAYS |   |   |   |    |    | NUMBER SURVIVALS |
|--------------------|-----------------|--------------------|-----------------|---|---|---|----|----|------------------|
|                    |                 |                    | 2               | 4 | 6 | 8 | 10 | 14 |                  |
| Sulfanilamide      | 3               | 0.020              | 3               | 3 | 3 | 2 | 2  | 0  | 0                |
| Sulfanilamide      | 3               | 0.050              | 3               | 3 | 2 | 1 | 1  | 0  | 0                |
| Sulfanilamide      | 3               | 0.100              | 3               | 3 | 3 | 2 | 2  | 0  | 0                |
| Sulfanilamide      | 3               | 0.200              | 3               | 3 | 3 | 2 | 1  | 0  | 0                |
| Neoprontosil       | 3               | 0.020              | 3               | 2 | 2 | 1 | 0  | 0  | 0                |
| Neoprontosil       | 3               | 0.050              | 3               | 3 | 2 | 2 | 1  | 0  | 0                |
| Neoprontosil       | 3               | 0.100              | 3               | 3 | 3 | 2 | 1  | 0  | 0                |
| Neoprontosil       | 3               | 0.200              | 3               | 2 | 2 | 1 | 0  | 0  | 0                |
| Untreated controls | 8               | -                  | 8               | 8 | 8 | 6 | 4  | 0  | 0                |

\*Administered once daily for five days in succession. Each dose of both compounds was given in 80 c.c. of 5 per cent solutions of glucose. Time required for injections varied from three hours and forty-seven minutes to four hours and two minutes. First dose of each compound was given twenty-four hours after inoculation.

†Weights 2,400 to 2,800 Gm. Inoculated intravenously with 0.8 c.c. of eighteen-hour broth culture of virulent beta hemolytic streptococcus.

In one experiment 32 rabbits were inoculated in this manner with 8 being kept as untreated controls. Of the remaining 24 animals 12 were given sulfanilamide and 12 neoprontosil intravenously by the drip method at daily intervals for five days in succession, the first dose of each compound being given twenty-four hours after inoculation, at which time positive heart blood cultures were observed. The daily doses of each compound varied from 0.020 to 0.200 Gm. per kilogram of weight, equivalent to 1.4 to 14.0 Gm. per 70 kg. of weight. Each dose was dissolved in 80 c.c. of a 5 per cent solution of glucose and given intravenously by the drip method, each treatment requiring about four hours. No difficulties were encountered in giving the injections by the gravity method

through the veins of the ears, the animals being confined in a special box in a comfortable position without the need of employing anesthesia. The results are summarized in Table I.

A second series of 32 rabbits was inoculated in the same manner, 8 being kept as untreated controls, and the remaining 24 treated with sulfanilamide and neoprontosil in the same dosage per kilogram of weight except that each compound was administered daily by intravenous injection by the syringe method at the rate of 5 c.c. per minute. Sulfanilamide was employed in an 0.8 per cent solution which required the injection of from 6 to about 48 c.c. in order to administer doses of 0.020 to 0.200 Gm. per kilogram of weight. In the case of neoprontosil a stock 5 per cent solution was employed so diluted with sterile water that each dose was administered in a volume of 15 c.c. The results are summarized in Table II.

TABLE II

RESULTS OF TREATMENT OF RABBITS WITH SULFANILAMIDE AND NEOPRONTOSIL ADMINISTERED BY THE SYRINGE METHOD\*

| COMPOUNDS          | NUMBER RABBITS† | DOSE PER KG. (GM.) | SURVIVALS: DAYS |   |   |   |    |    | NUMBER SURVIVALS |
|--------------------|-----------------|--------------------|-----------------|---|---|---|----|----|------------------|
|                    |                 |                    | 2               | 4 | 6 | 8 | 10 | 14 |                  |
| Sulfanilamide      | 3               | 0.020              | 3               | 3 | 2 | 1 | 0  | 0  | 0                |
| Sulfanilamide      | 3               | 0.050              | 3               | 3 | 3 | 2 | 0  | 0  | 0                |
| Sulfanilamide      | 3               | 0.100              | 3               | 3 | 3 | 3 | 3  | 0  | 0                |
| Sulfanilamide      | 3               | 0.200              | 3               | 3 | 3 | 2 | 1  | 0  | 0                |
| Neoprontosil       | 3               | 0.020              | 3               | 3 | 3 | 2 | 0  | 0  | 0                |
| Neoprontosil       | 3               | 0.050              | 3               | 3 | 3 | 2 | 0  | 0  | 0                |
| Neoprontosil       | 3               | 0.100              | 3               | 3 | 3 | 2 | 1  | 0  | 0                |
| Neoprontosil       | 3               | 0.200              | 3               | 3 | 3 | 3 | 2  | 0  | 0                |
| Untreated controls | 8               | -                  | 8               | 8 | 7 | 6 | 3  | 0  | 0                |

\*Administered once daily for five days in succession. Each dose of sulfanilamide was dissolved in 6 to 48 c.c. of 5 per cent solutions of glucose. Each dose of neoprontosil was prepared of a stock 5 per cent solution diluted with sterile distilled water to a total volume of 15 c.c. First dose of each compound was given twenty-four hours after inoculation.

†Weights 2,000 to 2,400 Gm. Inoculated intravenously with 0.8 c.c. of eighteen-hour broth culture of virulent beta hemolytic streptococcus.

Determinations of free sulfanilamide in the blood were made about one hour after the completion of the second treatment by both the drip and syringe methods of administration, and about two hours after the fourth treatment by both methods. The average results are summarized in Table III. It will be noted that the concentrations after the syringe method of administration were frequently higher than after the continuous drip method of administration, probably because of greater elimination of both compounds during the four hours required for their administration by the latter method.

#### TOXICITY OF SULFANILAMIDE AND NEOPRONTOSIL BY INTRAVENOUS ADMINISTRATION

*Toxicity of Sulfanilamide.*—Sulfanilamide was well borne by both methods of administration. All of four normal rabbits given the compound in doses of 0.020, 0.050, 0.100, and 0.200 Gm. per kilogram, respectively, at daily intervals for five days in succession by the intravenous drip method in the same manner as employed in the therapeutic experiments, survived indefinitely, with no clinical evidences of toxicity. The same was true of four normal controls given the

compound in similar dosage daily for five days in succession by the syringe method of intravenous injection with no evidences of the "speed shock" observed by Hirschfeld, Hyman, and Wanger<sup>9</sup> in relation to the intravenous injection of the organic arsenical and other compounds, probably because the sulfanilamide was administered in an 0.8 per cent solution.

TABLE III  
CONCENTRATIONS OF FREE SULFANILAMIDE IN THE BLOOD\*

| COMPOUNDS     | DOSE PER KG.<br>(GM.) | METHOD OF AD-<br>MINISTRATION | AVERAGE BLOOD CONCENTRA-<br>TION AFTER |          |
|---------------|-----------------------|-------------------------------|--|----------|
|               |                       |                               | 2ND DOSE                               | 4TH DOSE |
| Sulfanilamide | 0.020                 | Drip                          | 1.1                                    | 3.1      |
| Sulfanilamide | 0.020                 | Syringe                       | 2.2                                    | 1.2      |
| Sulfanilamide | 0.050                 | Drip                          | 3.5                                    | 3.1      |
| Sulfanilamide | 0.050                 | Syringe                       | 1.6                                    | 1.2      |
| Sulfanilamide | 0.100                 | Drip                          | 4.0                                    | 3.7      |
| Sulfanilamide | 0.100                 | Syringe                       | 1.5                                    | 1.6      |
| Sulfanilamide | 0.200                 | Drip                          | 12.05                                  | 5.3      |
| Sulfanilamide | 0.200                 | Syringe                       | 7.1                                    | 5.6      |
| Sulfanilamide | 0.020                 | Drip                          | 0.5                                    | 0        |
| Neoprontosil  | 0.020                 | Syringe                       | 1.5                                    | 0.8      |
| Neoprontosil  | 0.050                 | Drip                          | 0.5                                    | 0.5      |
| Neoprontosil  | 0.050                 | Syringe                       | 0.5                                    | 0.5      |
| Neoprontosil  | 0.100                 | Drip                          | 0                                      | 0.5      |
| Neoprontosil  | 0.100                 | Syringe                       | 0.5                                    | 0.8      |
| Neoprontosil  | 0.200                 | Drip                          | 0.8                                    | Negative |
| Neoprontosil  | 0.200                 | Syringe                       | 3.6                                    | 1.8      |

\*Milligrams per 100 c.c. Determinations were made about one hour after the completion of injections by the drip method and about two hours after injections by the syringe method.

*Toxicity of Neoprontosil.*—Of particular interest, however, was the fact that neoprontosil was likewise well borne by intravenous injection. All of four normal rabbits given 0.020, 0.050, 0.100, and 0.200 Gm. per kilogram, respectively, at daily intervals for five days in succession by the intravenous drip method in the same manner as employed in the therapeutic tests, survived indefinitely with no clinical evidences of toxicity. The same was true of four additional animals given the compound in the same dosage daily for five days in succession by the syringe method of administration, each dose being diluted with sterile water to a total volume of 15 c.c.

However, since neoprontosil is not customarily administered to human beings by intravenous injection, we have thought it advisable to conduct additional toxicity tests by this route of parenteral administration.

For this purpose normal rabbits were given neoprontosil\* intravenously in 5 per cent solution twice daily (10 A.M. and 3 P.M.) for eight days in succession in doses of 0.1, 0.2, 0.5, and 1.0 Gm. per kilogram by syringe injection, two animals being used for each dose. The total daily dosage varied, therefore, from 0.2 to 2.0 Gm. per kilogram or from 1.6 to 16.0 Gm. per kilogram over the eight days of administration and equivalent to from 14.0 to 140.0 Gm. per day for a human adult weighing 70 kg. As shown in Table IV, all animals survived indefinitely except one which died after six doses of 1.0 Gm. each or a total of 6.0 Gm. per kilogram over three days of administration.

\*We are indebted to the Department of Medical Research, Winthrop Chemical Company, for kindly supplying the compound.

All surviving animals were killed one week after the last dose and microscopic examinations were made of the liver, spleen, kidneys, and sternal bone marrow of each. No pathologic tissue changes were found in the sections of the spleens or bone marrow. The livers of two animals showed slight cloudy swelling with perilobular congestion, but no fatty degeneration or necrosis of the peripheral lobular cells. The kidneys of only one animal showed some dilatation of the convoluted or collecting tubules associated with tubular necrosis of minor degree. The histopathologic changes, therefore, were much less marked than observed by Rake, VanDyke, and Corwin<sup>10</sup> following the oral administration of sulfathiazole and sulfapyridine to mice, rats, and monkeys and also less than observed by Kolmer, Rule, and Groskin<sup>11</sup> following the oral administration of sulfathiazole and sulfathiazoline to rabbits.

TABLE IV  
TOXICITY OF NEOPRONTOSIL FOR RABBITS BY INTRAVENOUS ADMINISTRATION

| WEIGHT<br>(GM.) | GM. PER<br>KG.<br>TWICE<br>DAILY* | TOTAL<br>PER KG.<br>(GM.) | RESULTS      | BLOOD CONCENTRATIONS (MG. PER 100 C.C.)† |          |          |          |           |
|-----------------|-----------------------------------|---------------------------|--------------|--|----------|----------|----------|-----------|
|                 |                                   |                           |              | 1ST DOSE                                 | 3RD DOSE | 5TH DOSE | 9TH DOSE | 15TH DOSE |
| 2,000           | 0.1                               | 1.6                       | Survived     | 0.16                                     | 0.12     | 0.16     | 0.16     | 0.14      |
| 1,950           | 0.1                               | 1.6                       | Survived     | Negative                                 | Negative | 0.08     | 0.08     | 0.04      |
| 1,850           | 0.2                               | 3.2                       | Survived     | 0.16                                     | 0.16     | 0.24     | 0.24     | 0.14      |
| 1,900           | 0.2                               | 3.2                       | Survived     | Negative                                 | 0.12     | 0.16     | 0.16     | 0.14      |
| 1,750           | 0.5                               | 8.0                       | Survived     | 0.28                                     | 0.32     | 0.36     | 0.40     | 0.32      |
| 1,800           | 0.5                               | 8.0                       | Survived     | 0.28                                     | 0.28     | 0.36     | 0.38     | 0.32      |
| 1,700           | 1.0                               | 6.0                       | Died 4th day | 0.40                                     | 0.72     | 0.60     | 0        | 0         |
| 1,700           | 1.0                               | 16.0                      | Survived     | 0.50                                     | 0.56     | 0.56     | 0.60     | 0.64      |

\*At 10 A.M. and 3 P.M. for eight days in succession; 5 per cent solution of neoprontosil was employed.

†Determinations were made four hours after administration.

Total erythrocyte and leucocyte counts were made just before and four hours after the second, sixth, tenth, and sixteenth doses in each rabbit. Allowing for physiologic variations in different animals, the results have shown no effect upon the erythrocytes and leucocytes of those given 0.2 and 0.4 Gm. per kilogram per day. In those animals given 1.0 and 2.0 Gm. per kilogram per day a slight but definite decrease of the total erythrocytes per cubic millimeter of blood was noted, especially in the case of the animal that died after six doses of 1.0 Gm. each per kilogram, in which the total erythrocytes had dropped from 6,070,000 per cubic millimeter before the first dose to 4,200,000 four hours after the sixth dose. None of the animals, including that which died, showed a leucopenia; on the contrary, most of them showed a tendency to slight leucocytosis.

Blood examinations for the determination of free sulfanilamide were made four hours after the first, third, fifth, and fifteenth injections of the compounds in each rabbit. As shown in Table IV, the concentrations were quite low, evidently due in part to rapid acetylation, and usually much less than those observed one hour after administration, shown in Table III.

Agglutination tests in vitro, employing 1 c.c. of a 1 per cent suspension of washed human erythrocytes with amounts of 5 per cent solution of neoprontosil, varying from 0.1 to 1.0 c.c., and the addition of physiologic saline solution to a total volume of 2 c.c. showed some agglutination (+ to +++) upon microscopic examination of each mixture after incubation in a water bath for two hours at

37° C., but in spite of this evidence of the agglutination of washed erythrocytes *in vitro* no clinical evidences of intravascular agglutination or hemolysis were apparent *in vivo* in the case of rabbits, probably because of the protection afforded by the plasma proteins.

#### THERAPEUTIC RESULTS IN THE TREATMENT OF RABBITS

As shown in Tables I and II, both sulfanilamide and neoprontosil administered once daily for five days in succession by the intravenous drip and syringe methods of administration proved ineffective in the treatment of rabbits inoculated intravenously with virulent beta hemolytic streptococcus. All treated animals, including 16 untreated controls, died within a period of fourteen days. All developed suppurative arthritis with positive joint cultures at the time of death.

These results were not unexpected in the case of those animals given the compounds intravenously once a day by syringe injection in view of the unfavorable results previously observed following the oral administration of sulfanilamide to rabbits developing this type of streptococcus infection.<sup>7</sup> The main purpose of the investigation was to determine whether or not better results could be secured by the intravenous drip method of administration, but such has not been observed in these experiments. It is likely that failure of treatment by both methods of administration was largely due to the lack of maintenance of therapeutically effective concentrations of free sulfanilamide in the blood because of rapid acetylation of sulfonamide compounds by rabbits, along with the fact that only single doses were given once daily for five days in succession. It was hoped, however, that the intravenous drip method of administration, covering a period of about four hours for the injection of each dose, might prove superior, but such has not been observed.

#### THERAPEUTIC RESULTS IN THE TREATMENT OF HUMAN BEINGS

Both sulfanilamide and neoprontosil, however, may be safely administered to human beings by the intravenous drip method. The same is true of the soluble sodium salt of sulfapyridine. At least one of us (J. A. K.) has administered sulfanilamide by this method to five adult patients with severe surgical hemolytic streptococcus septicemia in whom oral administration was unsatisfactory because of nausea and vomiting. There were no apparent ill effects. Each treatment consisted in the intravenous injection of a mixture of 600 c.c. of 0.8 per cent solution of sulfanilamide in sterile saline solution diluted with 600 c.c. of 5 per cent solution of glucose at the rate of about 200 c.c. per hour, covering a period of about six hours. This administered 4.8 Gm. and the injections were repeated at intervals of six hours (two per twenty-four hours, totaling 9.6 Gm.) for five days in succession. The therapeutic effects were very prompt and all recovered.

Three additional cases of postabortal hemolytic streptococcus septicemia were treated in the same manner with neoprontosil with no apparent ill effects and with complete recoveries. Each treatment consisted of the intravenous drip administration of 100 c.c. of a 5 per cent solution of neoprontosil diluted with 1,100 c.c. of 5 per cent glucose solution given at the rate of about 200 c.c. per

hour over a period of about six hours (5 Gm.). The injections were repeated at intervals of six hours (two per twenty-four hours, totaling 10 Gm.) for five days in succession. It is true that neoprontosil has not been customarily administered by intravenous injection, since it is stated that this frequently produces nausea, vomiting, and immediate bowel movements,<sup>12</sup> but such were not observed following the intravenous drip administration of the compound in our three cases by the method employed.

Sodium sulfapyridine has been given to two adult patients with subacute bacterial endocarditis due to infection with *Streptococcus viridans* with two to four positive blood cultures, respectively, before the institution of treatment. Each treatment consisted of the intravenous drip administration of 4 Gm. of the compound dissolved in 1,200 c.c. of sterile saline solution at the rate of about 200 c.c. per hour over a period of about six hours. The injections were repeated at intervals of six hours (two per twenty-four hours, totaling 8 Gm.) for eight days in succession. There were no ill effects, although one patient developed a very mild hematuria on the sixth day of treatment which may have been due to the compound. At the completion of the course treatment was continued for twenty-four days by oral administration of sulfapyridine combined with the intravenous injection of typhoid-paratyphoid vaccine every three days for eight doses, for the production of fever. In spite of temporarily negative blood cultures, both patients ultimately died of the disease.

#### SUMMARY

1. The intravenous drip administration of sulfanilamide and neoprontosil in doses of 0.020 to 0.200 Gm. per kilogram once a day for five days in succession was well borne by rabbits with no evidences of toxicity.

2. The intravenous injection of neoprontosil to rabbits by syringe in doses of 0.1 to 1.0 Gm. per kilogram twice daily for eight days in succession was well borne with no clinical evidences of toxicity.

3. The intravenous drip administration of sulfanilamide in dose of 4.8 Gm. (600 c.c. of 0.8 per cent solution diluted with 600 c.c. of 5 per cent glucose solution) at the rate of 200 c.c. per hour for six hours, repeated every six hours (two injections per twenty-four hours) for five days in succession, was well tolerated by human beings with hemolytic streptococcus septicemia and gave excellent therapeutic results.

4. The intravenous drip administration of neoprontosil in dose of 5 Gm. (100 c.c. of 5 per cent solution diluted with 1,100 c.c. of 5 per cent glucose solution) at the rate of 200 c.c. per hour for six hours, repeated every six hours (two injections per twenty-four hours) for five days in succession, was well tolerated by human beings with hemolytic streptococcus septicemia and gave excellent therapeutic results.

5. The intravenous administration of sodium sulfapyridine in dose of 4 Gm. dissolved in 1,200 c.c. of physiologic saline solution at the rate of 200 c.c. per hour for six hours (two injections per twenty-four hours) for eight days in succession was well tolerated by two cases of subacute bacterial endocarditis due to infection with *Streptococcus viridans* but with unsatisfactory therapeutic results.



6. The intravenous drip and syringe methods of administration of sulfanilamide and neoprontosil in doses of 0.020 to 0.200 Gm. per kilogram of weight once a day for five days in succession proved ineffective in the treatment of rabbits inoculated intravenously with virulent beta hemolytic streptococcus and developing suppurative arthritis with septicemia. Failure was largely ascribed to rapid acetylation of the compounds resulting in therapeutically ineffective concentrations of free sulfanilamide in the blood.

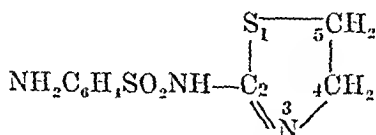
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# THE CHEMOTHERAPEUTIC STUDIES OF 2-SULFANILYL-3-5-DIHYDROTHIAZOLE (SULFATHIAZOLINE)<sup>a</sup>

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SULFATHIAZOLINE,<sup>1</sup> chemically 2-(p-aminobenzenesulfonyl)aminothiazoline,



differs from sulfathiazole in that the double bond in the latter, between carbon atoms 4 and 5, is saturated by the addition of an hydrogen atom to each of these carbon atoms. It is a colorless, crystalline compound, sparingly soluble in water, soluble in dilute mineral acids and alkalis. It has a melting point of 207 to 209° C.

## TOXICITY

Mice tolerate more than 20 Gm. per kg. of body weight of sulfathiazoline given orally (Table I).

TABLE I

TOXICITY OF SULFATHIAZOLINE AND SULFATHIAZOLE ADMINISTERED TO MICE PER OS  
Drugs Given in a 10 Per Cent Gum Acacia Suspension

| DRUG            | DOSE PER KG.<br>(GM.) | NO. OF<br>ANIMALS | SURVIVALS |          |
|-----------------|-----------------------|-------------------|-----------|----------|
|                 |                       |                   | NO.       | PER CENT |
| Sulfathiazoline | 10.0                  | 20                | 20        | 100      |
|                 | 15.0                  | 25                | 25        | 100      |
|                 | 20.0                  | 50                | 49        | 98       |
| Sulfathiazole   | 10.0                  | 20                | 16        | 80       |
|                 | 15.0                  | 24                | 17        | 71       |
|                 | 20.0                  | 20                | 15        | 75       |

TABLE II

TOXICITY OF SULFATHIAZOLINE AND SULFATHIAZOLE ADMINISTERED TO RABBITS PER OS  
Drugs Given in Water Suspension

| DRUG            | DOSE PER KG.<br>(GM.) | NO. OF<br>ANIMALS | SURVIVALS |          |
|-----------------|-----------------------|-------------------|-----------|----------|
|                 |                       |                   | NO.       | PER CENT |
| Sulfathiazoline | 3.0                   | 12                | 12        | 100      |
|                 | 4.0                   | 22                | 19        | 86       |
| Sulfathiazole   | 4.0                   | 9                 | 5         | 66       |

According to Table II, sulfathiazoline was tolerated by 86 per cent of rabbits which received 4.0 Gm. per kilogram. Sulfathiazole, on the other hand, was tolerated by 66 per cent of rabbits which received 4.0 Gm. per kilogram.

<sup>a</sup>From the Dermatological Research Laboratories, Philadelphia, Division of Abbott Laboratories, North Chicago.

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Five dogs were given sulfathiazoline by mouth in a dose of 1.0 Gm. each daily for fifty days, and no diarrhea or vomiting was observed in any case. The dogs survived the fifty-day observation period and did not lose any weight (Table III).

TABLE III  
TOXICITY OF SULFATHIAZOLINE IN DOGS GIVEN ORALLY

| NO. OF DOG | WEIGHT IN LB.<br>WHEN TEST WAS<br>STARTED | WEIGHT IN LB.<br>WHEN TEST WAS<br>COMPLETED | DAILY DOSE<br>(GM.)* | TOTAL GIVEN<br>(GM.) |
|------------|---|---|----------------------|----------------------|
| 1          | 22 $\frac{1}{4}$                          | 24  | 1.0                  | 50†                  |
| 2          | 21  | 22 $\frac{1}{2}$                            | 1.0                  | 50                   |
| 3          | 22 $\frac{1}{2}$                          | 26  | 1.0                  | 50                   |
| 4          | 21  | 21 $\frac{1}{2}$                            | 1.0                  | 50                   |
| 5          | 20 $\frac{1}{4}$                          | 21 $\frac{1}{2}$                            | 1.0                  | 50                   |

\*Daily dose of 1.0 Gm. for 50 consecutive days.

†No diarrhea or vomiting was observed in any of the animals.

Kolmer<sup>1</sup> found that sulfathiazoline, in doses of 0.05, 0.1, and 0.2 Gm. per kilogram twice daily for ten days in succession (20 doses) by oral administration, was well tolerated by rabbits.

No macroscopic or microscopic injury of the meninges, brain, or spinal cord of any of the animals, including those receiving the largest amount of the compound, was found.

No gross or microscopic injury of the kidneys, liver, or spleen in a dose of 0.05 Gm. per kilogram twice daily for 20 doses was observed.

There was evidence of slight injury of the kidneys, liver, and spleen in the case of some animals given a dose of 0.1 Gm. per kilogram orally twice daily for 20 doses.

We made comparative studies of sulfathiazoline and sulfathiazole in reference to blood concentrations. The perusal of figures in test A shows a figure of 2.59 to 1.11 mg. per cent for sulfathiazoline. For sulfathiazole we found the blood concentration ranging from 3.2 to 1.19 mg. per cent. When a higher dose, namely, 0.5 Gm. per kilogram was given, the blood levels, two hours after injection, were considerably higher (Table IV).

*Therapeutic Effect in Pneumococcus Types II and III.*—In these experiments mice were infected intraperitoneally with 10 to 200 M.L.D. of types II or III pneumococcus, of which the average minimum lethal dose was 0.5 c.c. of 1:1,000,000 to 1:10,000,000 dilution of broth culture.

The drugs were given by mouth in a dose of 10 mg. three times daily at 9 A.M., 5 P.M., and 12 P.M. for five days; at 9 A.M. and 5 P.M. on the sixth day; and at 9 A.M. on the seventh day, a maximum of 18 treatments. From Table V, which presents a summary of the results obtained in several experiments, one should note that at the end of four days the percentage of survivals was higher for sulfathiazoline, 78 per cent against 73 per cent for sulfathiazole. At the end of the seventh day, however, the percentage of survivals is higher for sulfathiazole than for sulfathiazoline. The former shows 19 per cent survivals, and the latter, 14 per cent. At the end of twenty-eight days, 5 per cent of sulfathiazole-treated mice survived against 2 per cent for sulfathiazoline. In type III pneumococci infections the percentage of survivals is highest for sulfa-

TABLE IV  
BLOOD LEVELS IN MICE

| TEST | SULFATHIAZOLINE |  |                                  |       | SULFATHIAZOLE |  |                                  |       |
|------|-----------------|--|----------------------------------|-------|---------------|--|----------------------------------|-------|
|      | MOUSE NO.       | INTERVAL IN HOURS AT WHICH ANIMALS WERE KILLED | BLOOD LEVELS CALCULATED IN MG. % |       | MOUSE NO.     | INTERVAL IN HOURS AT WHICH ANIMALS WERE KILLED | BLOOD LEVELS CALCULATED IN MG. % |       |
|      |                 |  | FREE                             | CONJ. |               |  | FREE                             | CONJ. |
| A    | 1               | 2*   | 2.49                             | None  | 11            | 2*   | 3.20                             | None  |
|      | 2               | 2  | 2.59                             | None  | 12            | 2  | 2.91                             | None  |
|      | 3               | 4  | 1.56                             | 0.36  | 13            | 4  | 1.68                             | None  |
|      | 4               | 4  | 1.87                             | 0.26  | 14            | 4  | 1.24                             | None  |
|      | 5               | 6  | 1.40                             | None  | 15            | 6  | 1.42                             | None  |
|      | 6               | 6  | 1.18                             | None  | 16            | 6  | 1.30                             | None  |
|      | 7               | 8  | 1.30                             | None  | 17            | 8  | 1.60                             | None  |
|      | 8               | 8  | 1.11                             | None  | 18            | 8  | 1.19                             | None  |
|      | 9               | 24   | 1.19                             | None  | 19            | 24   | 1.67                             | None  |
|      | 10              | 24   | 1.24                             | None  | 20            | 24   | 1.36                             | None  |
| B    | 1               | 2 (After 1st dose)†                            | 5.71                             | 1.14  | 13            | 2 (After 1st dose)†                            | 15.88                            | 3.84  |
|      | 2               | 2 (After 1st dose)                             | 7.14                             | 2.28  | 14            | 2 (After 1st dose)                             | 7.66                             | 1.16  |
|      | 3               | 4 (After 1st dose)                             | 2.43                             | 1.21  | 15            | 4 (After 1st dose)                             | 5.29                             | 0.99  |
|      | 4               | 4 (After 1st dose)                             | 2.43                             | None  | 16            | 4 (After 1st dose)                             | 4.34                             | 1.58  |
|      | 5               | 6 (After 1st dose)                             | 2.86                             | None  | 17            | 6 (After 1st dose)                             | 6.71                             | None  |
|      | 6               | 6 (After 1st dose)                             | 2.14                             | 0.30  | 18            | 6 (After 1st dose)                             | 6.57                             | 0.76  |
|      | 7               | 15 (After 2nd dose)                            | 1.81                             | 1.05  | 19            | 15 (After 2nd dose)                            | 0.74                             | 0.30  |
|      | 8               | 15 (After 2nd dose)                            | 0.86                             | 0.28  | 20            | 15 (After 2nd dose)                            | 1.03                             | 0.29  |
|      | 9               | 2 (After 3rd dose)                             | 3.14                             | 1.29  | 21            | 2 (After 3rd dose)                             | 11.61                            | 4.63  |
|      | 10              | 2 (After 3rd dose)                             | 4.57                             | 0.86  | 22            | 2 (After 3rd dose)                             | 8.82                             | 5.88  |
|      | 11              | 4 (After 3rd dose)                             | 3.00                             | 3.08  | 23            | 4 (After 3rd dose)                             | 3.80                             | 0.60  |
|      | 12              | 4 (After 3rd dose)                             | 2.07                             | 1.14  | 24            | 4 (After 3rd dose)                             | 7.33                             | 1.15  |
| C    | 1               | 2‡   | 5.35                             | None  | 13            | 2‡   | 12.86                            | None  |
|      | 2               | 2  | 4.40                             | None  | 14            | 2  | 9.40                             | None  |
|      | 3               | 4  | 1.33                             | None  | 15            | 4  | 3.27                             | None  |
|      | 4               | 4  | 1.92                             | None  | 16            | 4  | 6.43                             | None  |
|      | 5               | 6  | 0.93                             | 0.31  | 17            | 6  | 3.63                             | None  |
|      | 6               | 6  | 0.88                             | 0.25  | 18            | 6  | 2.20                             | None  |
|      | 7               | 8  | 0.52                             | 0.25  | 19            | 8  | 0.45                             | 0.25  |
|      | 8               | 8  | 0.30                             | 0.25  | 20            | 8  | 0.72                             | 0.31  |
|      | 9               | 12   | 0.73                             | None  | 21            | 12   | 0.31                             | 0.28  |
|      | 10              | 12   | 0.30                             | None  | 22            | 12   | 0.33                             | 0.21  |
|      | 11              | 24   | 0.77                             | 0.73  | 23            | 24   | 0.78                             | 0.65  |
|      | 12              | 24   | 0.50                             | 0.75  | 24            | 24   | 0.48                             | 1.08  |

\*Each mouse received only one dose of 0.002 Gm., which is approximately 0.1 Gm. per kilogram of body weight, in 10 per cent gum acacia solution.

†Each dose administered was of 0.010 Gm. per os in a 10 per cent gum acacia solution.

1st dose given at 10 A.M.

2nd dose given at 4 P.M.

3rd dose given at 10 A.M. next day.

‡Each mouse received one dose of 0.010 Gm. per os in a 10 per cent gum acacia solution.

TABLE V  
THERAPEUTIC EFFECT IN TYPES II AND III PNEUMOCOCCIC INFECTION IN MICE

| TYPE OF PNEUMO-<br>COCCUS | DRUG            | NO. MICE<br>USED | PERCENTAGE OF SURVIVALS IN DAYS |     |    |    |    |    |    |    |    |    |  |  |
|---------------------------|-----------------|------------------|---------------------------------|-----|----|----|----|----|----|----|----|----|--|--|
|                           |                 |                  | 1                               | 2   | 3  | 4  | 5  | 6  | 7  | 14 | 21 | 28 |  |  |
| II                        | Sulfathiazoline | 170              | 100                             | 100 | 90 | 78 | 48 | 24 | 14 | 2  | 2  | 2  |  |  |
|                           | Sulfathiazole   | 105              | 100                             | 95  | 85 | 73 | 54 | 33 | 19 | 6  | 5  | 5  |  |  |
|                           | Controls        | 101              | 15                              | 0   |    |    |    |    |    |    |    |    |  |  |
| III                       | Sulfathiazoline | 31               | 100                             | 86  | 65 | 35 | 26 | 13 | 3  | 3  | 3  | 3  |  |  |
|                           | Sulfathiazole   | 33               | 94                              | 82  | 58 | 27 | 18 | 15 | 6  | 3  | 3  | 3  |  |  |
|                           | Controls        | 25               | 48                              | 48  | 8  | 0  |    |    |    |    |    |    |  |  |

thiazoline at the end of five days; i.e., 26 per cent for sulfathiazoline and 18 per cent for sulfathiazole. At the end of the twenty-eighth day the percentage of survivals is equal for both drugs.

#### THERAPEUTIC EFFECT IN STAPHYLOCOCCUS AUREUS INFECTIONS IN MICE

Mice were infected intravenously (saphenous vein) with 0.2 c.c. of a dilute saline suspension of F.D.A. strain No. 209 *Staphylococcus aureus*. Prior to the experiment the strain was passed through mice four times, the abscessed kidneys of the infected animals being removed at the time of death and recultured on plain agar. This increased the virulence of the strain so that 100 per cent of a series of at least 25 mice were killed within one to five days as a result of infection.

The mice were treated one and one-half, seven, twenty-four, and thirty-two hours after infection, then once daily for fourteen days, a total of 18 treatments per os. From Table VI one can see that the percentage of survivals for sulfathiazoline at the end of four days was 100; for sulfathiazole, 84. At the end of fourteen days the percentage of survivals for sulfathiazoline is higher than that for sulfathiazole—48 per cent as compared with 28 per cent. At the end of twenty-eight days 4 per cent of sulfathiazoline-treated mice survived as compared with 8 per cent of sulfathiazole.

TABLE VI  
THERAPEUTIC EFFECT IN STAPHYLOCOCCUS AUREUS INFECTIONS IN MICE

| DRUG            | NO. OF<br>MICE<br>USED | PERCENTAGE OF SURVIVALS, DAYS |     |     |     |    |    |    |    |    |    |
|-----------------|------------------------|-------------------------------|-----|-----|-----|----|----|----|----|----|----|
|                 |                        | 1                             | 2   | 3   | 4   | 5  | 6  | 7  | 14 | 21 | 28 |
| Sulfathiazoline | 25                     | 100                           | 100 | 100 | 100 | 96 | 88 | 80 | 48 | 4  | 4  |
| Sulfathiazole   | 25                     | 100                           | 88  | 88  | 84  | 80 | 76 | 72 | 28 | 16 | 8  |
| Controls        | 25                     | 100                           | 96  | 80  | 16  | 0  |    |    |    |    |    |

These results represent a summary of 5 individual experiments.

#### CONCLUSIONS

1. When tested on mice, rabbits, and dogs, sulfathiazoline proved to be lower in toxicity than sulfathiazole.
2. The therapeutic effect of sulfathiazoline in mice with type II and III pneumococcal infection was about the same as that observed with sulfathiazole.
3. The percentage of survivals of mice infected with *Staphylococcus aureus* and treated with sulfathiazoline was found to be higher in the first fourteen days of observation, while in periods of twenty-one and twenty-eight days sulfathiazole showed more survivals.

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## THE SURFACE AREA OF THE HUMAN ERYTHROCYTE\*

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IN THE study of the anemias it has been customary to consider the amount of hemoglobin, the number and size (volume and diameter) of the red blood cells, and the relationship between them. All this is based upon the assumption that the hemoglobin is evenly and diffusely distributed throughout the cell mass. However, evidence has been brought forth to show that the molecules of hemoglobin are deposited on the surface of the cell rather than within the cell body.<sup>10</sup> If this were the case, one might indeed imagine that this would increase the efficiency of hemoglobin as an oxygen carrier, its chief function. Emmons,<sup>2</sup> after determining and comparing the red blood cell count, volume, thickness, area, and the hemoglobin content of several species of animals, showed that, although these values varied considerably in different species, the total surface area of the red cells per unit volume of blood was remarkably constant in all of them. This fact also would favor the concept that hemoglobin is limited to the surface of the cell. The surface area then would seem to be an important factor in evaluating oxygen-carrying efficiency of the red blood cells in a case of anemia.

The earliest attempt to calculate the surface area of the red cell was by Burkner,<sup>2</sup> who used the formula  $A = \pi \frac{D^2}{2}$  ( $D$  representing the diameter), neglecting the thickness of the cell and assuming the cell to be flat. Values thus obtained were, on the face of it, untrue, since they were less than that of a sphere of the same volume—the form that displays the smallest area per volume.

If we assume the erythrocyte to be a short cylinder or flat disk, the area can be computed mathematically by a simple formula. Knowing the diameter, we can determine the top surface area by the formula  $\pi r^2$  ( $r$  representing radius). The area around the cell is equal to the circumference ( $2\pi r$ ) multiplied by the thickness. Since the volume equals the top area multiplied by the thickness, the latter can be computed by dividing the volume by the top area, or  $T = \frac{V}{\pi r^2}$  ( $T$  representing thickness and  $V$  representing volume). The total area is twice the area of the top surface plus that around the cell. The formula then would be  $A = 2\pi r^2 + 2\pi rT$  or  $A = 2\pi r (r + T)$ .

The red cell, however, has a characteristic curved configuration and so would exhibit a larger area. By the use of Guldin's theorems of Integral Calculus, Emmons<sup>2</sup> computed the surface area for several species of animals. The neces-

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sary values had to be obtained by actually constructing models of the red cells and taking direct measurements. Such a method is obviously a very laborious and impractical one. If the above-mentioned formula would give close enough results, we would have a very simple and practicable method.

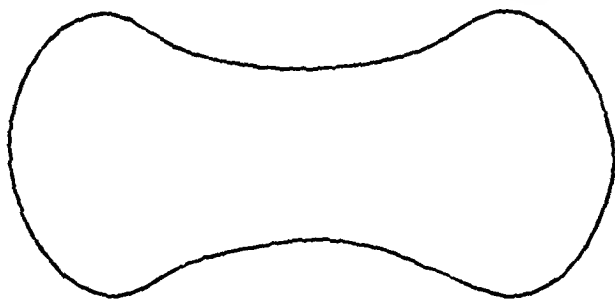


Fig. 1.

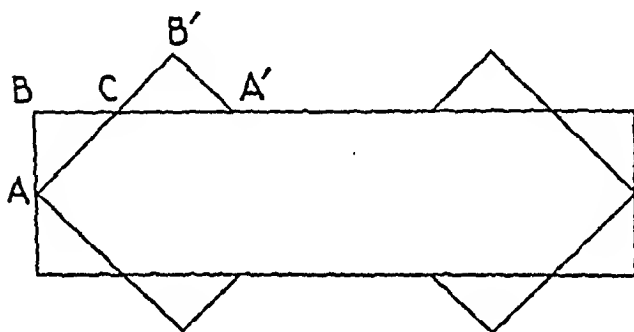


Fig. 2.

The object of this work is to develop a simple method of determining and establishing the normal value for the surface area of the human erythrocyte. This can then form a basis from which many of the hematologic relationships and implications can be investigated. We studied a series of 50 normal young adults (doctors, nurses, and hospital attendants), 27 males and 23 females.\* All the determinations were done on venous blood. The anticoagulant used was prepared according to the method of Wintrobe as follows: 0.6 Gm. of ammonium oxalate and 0.4 Gm. of potassium oxalate was dissolved in 100 c.c. of water. One cubic centimeter of this solution was placed in each test tube, and the tube was then dried in the oven. This amount of anticoagulant was used for 10 c.c. of blood. The hemoglobin was determined by the Haden-Hausser method (100 per cent = 15.8 Gm. per 100 c.c.). The hematocrit was determined by the Sanford-Magath method. Cell measurements were made with the filar micrometer measuring to within 0.5 microns. Thickness was computed by dividing the mean corpuscular volume by  $\pi r^2$ ,  $r$  being the mean radius of the cell. The formula used to compute the surface area was  $A = 2\pi r(r + T)$ ,  $T$  being the thickness.

Table I represents our figures and results. The surface area of the entire series of cases ranged from 129.09 to 142.56 square microns, the average being

\*Nine of the female subjects were nurses of the University Hospital, Ann Arbor, Mich. In these cases the hemoglobin was determined by the Sahli method and the hematocrit by the Wintrobe method.

TABLE I  
HEMATOLOGIC VALUES IN THIS STUDY

| NO. | NAME  | R.B.C.<br>COUNT<br>(MIL-<br>LIONS) | HB.<br>(GM.) | HEMATO-<br>CRIT<br>(C.C.) | MEAN<br>CORPUSC-<br>ULAR<br>VOLUME<br>(CUBIC<br>MICRONS) | DIAMETER<br>(MICRONS) | THICKNESS<br>(MICRONS) | SURFACE<br>AREA<br>(SQUARE<br>MICRONS) |
|-----|-------|------------------------------------|--------------|---------------------------|--|-----------------------|------------------------|--|
| 1   | L. K. | 4.53                               | 12.02        | 36.5                      | 80.6   | 7.46                  | 1.84                   | 130.45                                 |
| 2   | C. M. | 5.03                               | 14.11        | 44.0                      | 87.5   | 7.14                  | 2.18                   | 129.09                                 |
| 3   | E. L. | 4.48                               | 13.85        | 43.5                      | 97.1   | 7.04                  | 2.12                   | 142.56                                 |
| 4   | C. G. | 4.98                               | 13.06        | 42.5                      | 85.3   | 7.59                  | 1.88                   | 135.28                                 |
| 5   | M. U. | 4.72                               | 13.85        | 42.5                      | 90.0   | 7.73                  | 1.92                   | 140.10                                 |
| 6   | E. A. | 4.66                               | 13.77        | 40.0                      | 85.8   | 7.66                  | 1.86                   | 137.00                                 |
| 7   | M. F. | 4.81                               | 12.80        | 41.0                      | 85.2   | 7.62                  | 1.87                   | 135.86                                 |
| 8   | G. K. | 4.43                               | 12.93        | 40.0                      | 90.3   | 7.84                  | 1.87                   | 142.50                                 |
| 9   | M. F. | 4.45                               | 12.28        | 39.5                      | 88.8   | 7.13                  | 2.23                   | 129.35                                 |
| 10  | H. L. | 4.64                               | 13.00        | 43.0                      | 92.7   | 7.38                  | 2.17                   | 133.48                                 |
| 11  | M. H. | 4.32                               | 12.00        | 41.0                      | 94.9   | 7.43                  | 2.24                   | 138.84                                 |
| 12  | A. S. | 4.67                               | 13.00        | 42.0                      | 89.9   | 7.39                  | 2.10                   | 134.44                                 |
| 13  | E. K. | 4.63                               | 13.00        | 42.0                      | 90.7   | 7.37                  | 2.12                   | 134.62                                 |
| 14  | K. M. | 4.46                               | 12.50        | 41.0                      | 91.9   | 7.23                  | 2.24                   | 130.36                                 |
| 15  | M. H. | 4.51                               | 13.00        | 43.0                      | 95.3   | 7.35                  | 2.25                   | 136.70                                 |
| 16  | M. R. | 4.58                               | 12.50        | 41.0                      | 89.5   | 7.35                  | 2.10                   | 133.58                                 |
| 17  | J. M. | 4.52                               | 12.50        | 42.0                      | 92.9   | 7.26                  | 2.25                   | 133.91                                 |
| 18  | A. S. | 4.67                               | 13.00        | 44.0                      | 94.2   | 7.44                  | 2.17                   | 139.59                                 |
| 19  | L. B. | 4.32                               | 13.00        | 42.0                      | 97.2   | 7.54                  | 2.20                   | 140.18                                 |
| 20  | G. O. | 4.63                               | 13.00        | 42.0                      | 90.7   | 7.39                  | 2.11                   | 139.00                                 |
| 21  | D. C. | 4.45                               | 12.00        | 39.0                      | 87.6   | 7.19                  | 2.16                   | 129.85                                 |
| 22  | B. B. | 4.80                               | 13.50        | 44.0                      | 91.7   | 7.58                  | 2.02                   | 138.89                                 |
| 23  | M. R. | 4.84                               | 13.00        | 43.0                      | 88.8   | 7.13                  | 2.20                   | 129.85                                 |
| 24  | H. C. | 5.13                               | 14.50        | 47.0                      | 91.6   | 7.16                  | 2.27                   | 131.51                                 |
| 25  | K. C. | 4.74                               | 13.50        | 44.0                      | 92.8   | 7.42                  | 2.14                   | 136.51                                 |
| 26  | F. W. | 4.96                               | 14.00        | 47.0                      | 94.8   | 7.60                  | 2.10                   | 140.77                                 |
| 27  | A. A. | 5.07                               | 14.00        | 48.0                      | 94.7   | 7.20                  | 2.32                   | 133.84                                 |
| 28  | L. T. | 4.30                               | 12.50        | 40.0                      | 93.0   | 7.55                  | 2.10                   | 139.59                                 |
| 29  | A. K. | 4.64                               | 13.00        | 43.0                      | 92.7   | 7.26                  | 2.24                   | 133.84                                 |
| 30  | E. A. | 4.88                               | 14.50        | 46.0                      | 94.2   | 7.26                  | 2.28                   | 134.75                                 |
| 31  | N. W. | 4.68                               | 13.50        | 45.0                      | 96.1   | 7.26                  | 2.32                   | 135.81                                 |
| 32  | B. K. | 4.87                               | 13.50        | 45.0                      | 92.4   | 7.30                  | 2.20                   | 134.68                                 |
| 33  | S. M. | 4.91                               | 14.00        | 44.0                      | 89.6   | 7.20                  | 2.20                   | 131.14                                 |
| 34  | J. P. | 5.21                               | 15.00        | 50.0                      | 95.9   | 7.42                  | 2.22                   | 138.29                                 |
| 35  | I. P. | 5.12                               | 14.50        | 50.0                      | 97.7   | 7.58                  | 2.16                   | 141.80                                 |
| 36  | C. C. | 5.27                               | 15.50        | 48.0                      | 91.1   | 7.60                  | 2.01                   | 138.65                                 |
| 37  | E. S. | 5.31                               | 16.00        | 47.0                      | 88.7   | 7.28                  | 2.13                   | 131.90                                 |
| 38  | S. J. | 4.89                               | 13.50        | 45.0                      | 92.0   | 7.28                  | 2.20                   | 135.50                                 |
| 39  | H. B. | 5.12                               | 15.50        | 47.0                      | 91.8   | 7.25                  | 2.22                   | 133.28                                 |
| 40  | M. B. | 5.10                               | 14.00        | 48.0                      | 94.1   | 7.27                  | 2.26                   | 134.89                                 |
| 41  | W. B. | 5.04                               | 15.00        | 48.0                      | 95.2   | 7.35                  | 2.24                   | 136.63                                 |
| 42  | E. M. | 4.95                               | 14.00        | 46.0                      | 92.9   | 7.20                  | 2.28                   | 132.93                                 |
| 43  | D. L. | 5.28                               | 16.00        | 50.0                      | 94.7   | 7.19                  | 2.33                   | 134.06                                 |
| 44  | E. T. | 5.06                               | 15.00        | 49.0                      | 96.8   | 7.41                  | 2.25                   | 138.62                                 |
| 45  | H. S. | 5.02                               | 14.50        | 47.0                      | 93.6   | 7.37                  | 2.20                   | 135.56                                 |
| 46  | D. L. | 4.93                               | 14.00        | 44.0                      | 89.1   | 7.28                  | 2.14                   | 132.13                                 |
| 47  | F. B. | 4.61                               | 12.50        | 43.0                      | 93.2   | 7.41                  | 2.16                   | 136.50                                 |
| 48  | G. F. | 4.93                               | 14.50        | 46.0                      | 93.3   | 7.37                  | 2.21                   | 135.09                                 |
| 49  | G. L. | 4.85                               | 14.50        | 46.0                      | 94.8   | 7.62                  | 2.09                   | 140.45                                 |
| 50  | R. M. | 4.72                               | 13.00        | 43.0                      | 95.2   | 7.20                  | 2.34                   | 134.29                                 |

135.44, with a standard deviation of 3.517. In 80 per cent of our cases the values ranged from 130 to 140 square microns. Table II represents the distribution of the cases for the various values obtained in our series. The value obtained by Emmons,<sup>2</sup> using this very exact but very laborious method described above, was 144.8 square microns.



We recognize the fallacies of our method. It is based on the assumption that the erythrocyte is a flat disk, while it is known that it has a curved configuration. But perhaps the fallacy is not quite as great as it may appear to be. Fig. 1 represents a cross section of the normal configuration of the red blood cell. Let us, for the sake of discussion, exaggerate the curves to the point of making straight lines to assume the appearance of Fig. 2. The rectangular figure superimposed is the flat disk used in our computations. The area A'B'C, omitted in our calculations, would be compensated for by the area A B C.

TABLE II

DISTRIBUTION OF CASES ACCORDING TO COMPUTED SURFACE AREA OF ERYTHROCYTES

| SURFACE AREA IN SQUARE MICRONS | NUMBER OF CASES |
|--------------------------------|-----------------|
| 129                            | 4               |
| 130                            | 2               |
| 131                            | 3               |
| 132                            | 2               |
| 133                            | 7               |
| 134                            | 7               |
| 135                            | 6               |
| 136                            | 4               |
| 137                            | 1               |
| 138                            | 6               |
| 139                            | 2               |
| 140                            | 1               |
| 141                            | 1               |
| 142                            | 2               |

We feel deeply indebted to Dr. Raphael Isaacs, formerly associate professor of medicine, University of Michigan, School of Medicine, Ann Arbor, Mich., for his guidance and valuable suggestions in carrying out this work.

To summarize, a practicable technique for determining the mean surface area of the human erythrocyte with the normal value and range is presented. By this method the average total surface area of the normal human erythrocyte is  $135.44 \pm 3.517$  square microns. Eighty per cent of the cases ranged between 130 and 140 square microns. These results compare favorably with those obtained by more exact and much more difficult methods. This procedure opens possibilities for further investigations into the interrelationships of the surface area with other hematologic values and into the question of the distribution of hemoglobin in the red blood cell.

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# SARCOIDOSIS

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## A CONSIDERATION OF THE CLINICAL AND HISTOLOGIC CRITERIA DIFFERENTIATING SARCOIDOSIS FROM TUBERCULOSIS

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### INTRODUCTION

JONATHAN HUTCHINSON<sup>1</sup> in 1875 was the first to call attention to a peculiar malady of the skin in a woman named Mortimer which was characterized by granulomatous infiltration and which he referred to as Mortimer's malady. Besnier,<sup>2</sup> fourteen years later, reported similar and more detailed clinical observations of this disease, but it was not until 1899 that Caesar Boeck<sup>3</sup> recorded the pathologic histology of these granulomatous lesions and described an associated lymphadenopathy. Many subsequent investigators frequently mentioned that the cutaneous lesions were accompanied by widespread adenopathy, dactylitis, and roentgenographic changes in the fingers and toes, yet Kuznitzky and Bittorf<sup>4</sup> in 1915 were the first to demonstrate conclusively diffuse sarcoid lesions and to establish definitely Boeck's sarcoidosis as a generalized pathologic process. Although their observations have been corroborated many times, the disseminated character of the disease is still far too often not appreciated.

Practically every organ in the body may be involved, but lesions occur most frequently in the skin, lymph nodes, bones of the hands and feet, and in the lungs. The skin eruption, as described by Boeck, is usually widespread, symmetrical, nodular, pigmented, and infiltrative but never ulcerative. It affects usually the face, ears, nose, and extensor surfaces of the extremities. The adenopathy may be generalized or limited, superficial or deep seated; it often occurs in persons who never develop lesions in the skin. When the enlargement of the lymph nodes is confined to a single group, the condition is usually diagnosed as tuberculosis and may even be considered Hodgkin's disease or lymphosarcoma. Frequently the earliest signs and symptoms are referable to the lungs where roentgenologically there may be observed a marked bilateral and symmetrical enlargement of the hilar and peribronchial lymph nodes, a soft infiltration resembling caseous pneumonic tuberculosis, and often a peculiar basilar infiltration suggesting miliary tuberculosis. In spite of such marked roentgenographic involvement, the clinical manifestations of pulmonary disease are exceedingly few, since only a mild febrile reaction accompanies transitory chest pains and a slight nonproductive cough. Hemoptysis does occur, but it is rare.

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The alterations in the bones of the hands and feet have been described adequately roentgenologically by Kienhoek<sup>5</sup> and Jijngling<sup>6</sup> as punched out or reticulated areas of rarefaction in the medulla of the phalanges, with no involvement of the periosteum and joints.

In extensive studies of the blood Harrell<sup>7</sup> found definite increases in the content of calcium, protein, and phosphatase, but normal values for nonprotein nitrogen and phosphorus. In his series of cases Bence-Jones protein was usually present. In addition there were an increased sedimentation rate of the erythrocytes, a low or normal white blood cell count with neutropenia, eosinophilia, and relative monocytosis.

The disease occurs usually in early adult life and is generally benign, but it has been the cause of death in a few instances.<sup>8,9</sup> In Longcope and Pierson's<sup>10</sup> group and in Harrell's series Negroes predominated. Although for years it has been felt that the disease was related to tuberculosis, no investigator has been able to isolate the tubercle bacillus from the lesion or through animal inoculation. At present it is almost universally agreed that the etiology remains obscure. That the disease does occur without any abnormalities of the skin has been demonstrated by many observers. It was absent in three of Harrell's eleven cases, while 50 per cent of those published by Longcope and Pierson failed to show cutaneous lesions. We are presenting three patients with Boeck's sarcoidosis, in none of whom at any time did skin manifestations appear. In two of our cases the lymph nodes in localized regions only were clinically affected, while in the third case there was widespread involvement of the lymph nodes, and in addition lesions of the liver and lungs. The failure of clinicians to consider Boeck's sarcoidosis in the differential diagnosis of the lymphadenopathies and also the failure of roentgenologists to include this disease in the differential diagnosis of pulmonary changes resembling tuberculosis, but which cannot be proved to be such by any of the many laboratory procedures now available for this purpose, are emphasized. Moreover, these cases show how easily the histologic picture may be misinterpreted by the pathologist who is not familiar with it.

#### CASE HISTORIES

CASE 1.—K. G., a 38-year-old white woman, had at the age of 12 years a lump in her neck which first appeared at the angle of the jaw on the right side. It reached the diameter of 1 cm.; it was painless and unassociated with any systemic manifestations, such as fever, loss of weight, etc. This lump persisted apparently unchanged for twenty years, until at the age of 32 it began to enlarge without any detectable lymphadenopathy elsewhere. At that time there were no accompanying signs of systemic disease, yet a tonsillectomy was deemed advisable and was performed six months later, on March 28, 1935. Because no appreciable effect was noted upon the enlarging mass in the neck following the operation, the mass was extricated on April 29, 1941. At operation a firm, encapsulated oval mass, 3 by 2 cm., was found lying beneath the superficial fascia of the neck, near the right carotid artery at the angle of the jaw. No alterations in the near-by lymph nodes were found, and nodes were not palpable elsewhere.

Upon gross section the incised mass appeared to be composed of lymphoid tissue surrounded by a well-defined capsule. The cut surface was nodular and did not conform exactly to the picture of a merely hyperplastic lymph node. Microscopic examination revealed a lymph node, largely occupied by tubercle-like lesions composed of epithelioid cells among which there were small giant cells, not arranged in typical tubercles. At no place could

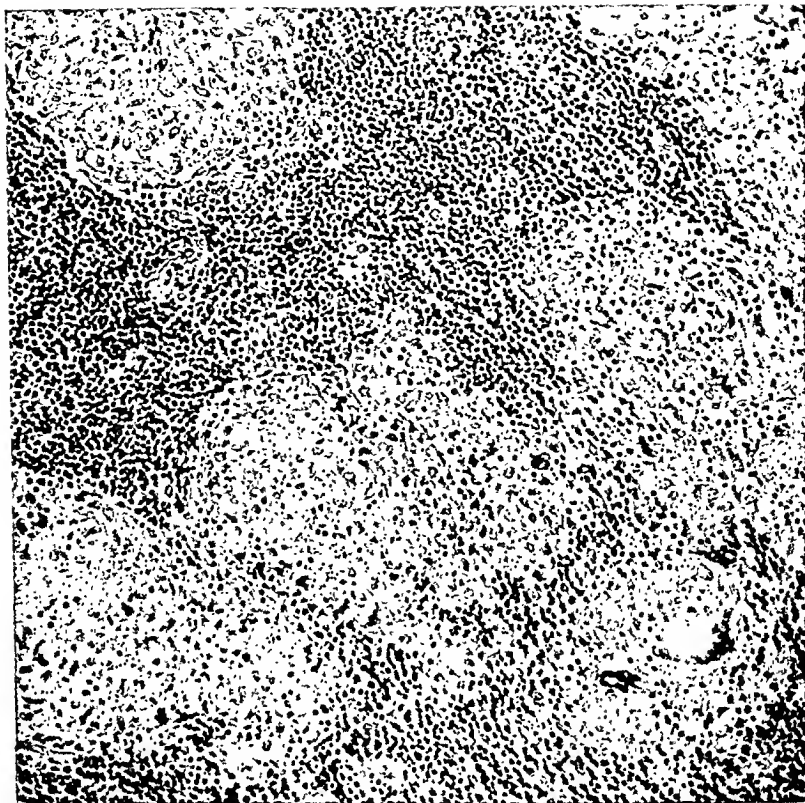


Fig. 1.

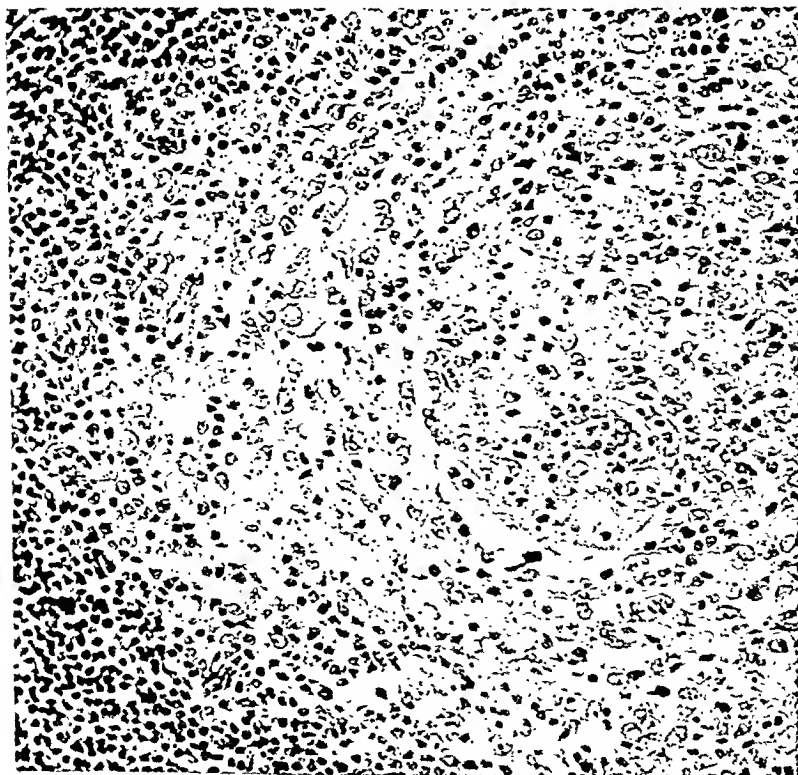


Fig. 2.

caseation be found. Minute foci of necrosis were present but extended search resulted in failure to demonstrate any acid-fast bacilli. The changes, on the other hand, were typical of those found in Boeck's sarcoidosis (Fig. 1). These findings stimulated roentgenologic examinations of the chest and of the bones of the hands and feet, yet no evidence of generalized Boeck's sarcoidosis could be found. The skin reaction to tuberculin in a dilution of 1:1,000 was negative, and there was never any drainage from the biopsy incision. Finally, the tonsils removed five years previously were re-examined in a series of sections, and a single sarcoid in one tonsil was found which was not evident in the routine sections (Fig. 2). Unfortunately we cannot say whether or not this is the tonsil from the same side as the excised lymph node from the neck.



Fig. 3.

CASE 2.—J. C., a 27-year-old white man, a member of the Kingston Fire Department, had noted a swelling of the right axillary region for six months; the mass was not painful. He was never "feverish" and had not lost weight. On November 15, 1939, a mass, 6 by 5 by 3 cm., was excised as well as three smaller oval structures resembling lymph nodes. All of these masses were well encapsulated and upon gross section showed slightly yellowish, minutely lobulated cut surfaces without evidence of softening or caseation. Microscopically, numerous lesions resembling so-called "hard tubercles" were found, and these consisted of large epithelioid cells in clusters but were devoid of typical Langhan's cells or caseation which characterize true tubercles. A few of these lesions did contain giant cells. No acid-fast bacilli could be found in the lesions, but in spite of these observations recorded at the time of the original examination, a diagnosis of tuberculous lymphadenitis was made. Not until one year later was the diagnosis questioned by the attending surgeon, who failed to observe other clinical signs or symptoms of an acid-fast infection. Upon re-examination of the sections in the light of recently acquired experience with the lesions of Boeck's sarcoidosis, it became clear at once that the original diagnosis was in error and that the changes were typical of the latter condition (Fig. 3).

Subsequent to the submission of a revised pathologic report, roentgenologic examination of the chest and of the bones of the hands and feet failed to demonstrate any changes, including those of generalized Boeck's sarcoidosis, and intradermal tuberculin in a dilution of 1:1,000 gave no reaction.



Fig. 4.

CASE 3.—G. E., a colored man, 31 years old, had been in excellent health all his life except for the usual childhood diseases until he became aware of an increasing dyspnea upon exertion early in January, 1935. Finally the dyspnea became so marked when at rest that it was necessary to hospitalize him on January 16. Upon admission to the Kingston Hospital the positive findings were those of marked dyspnea while lying in bed, pain and tenderness over the abdomen, more particularly in the gall bladder region, and changes in the lungs revealed only by roentgenograms. These latter changes were thought to be the result of either bronchopneumonia or miliary tuberculosis, in spite of normal temperature, no increase in the pulse rate, and the failure to demonstrate specific incitant organisms in the sputum after exhaustive search. There was a normal blood picture with perhaps a slight relative lymphocytosis. During two weeks' stay in the hospital he was afebrile, the pain in the abdomen subsided, and the dyspnea was relieved. A few days after discharge the pains recurred and were accompanied by "indigestion." The liver was now slightly enlarged and exquisitely tender. Upon readmission to the hospital the temperature was 100.2° F. and the pulse rate had increased to 108. No evidence of disease of the gall bladder and gastrointestinal tract was shown by x-ray studies and many other laboratory procedures. He became more comfortable upon the administration of sedatives and was discharged a few days later with a diagnosis of hepatitis.

During another admission in March of this same year an exploratory laparotomy yielded no additional information. The liver was large and bluish gray, and the gall bladder was

drained. Recovery from the operation was uneventful, but because of persistent changes in the lungs suggestive of tuberculosis, the patient was referred to the out-patient department of the Ulster County Tuberculosis Hospital. Later, owing to the difficulty in confirming a diagnosis of tuberculosis, he was admitted to this hospital for more intensive study. The observations of Dr. George Weber were most interesting: "The physical findings and x-ray plates (Fig. 4) seem to show a tuberculous process of the caseous-pneumonic type, involving chiefly the middle and lower lobes of the right lung with some spread to the left side. An enlarged mediastinal shadow, which looks like tumor growth, cannot be explained on the basis of tuberculous involvement in an adult." The sputum never yielded acid-fast bacilli, and several guinea pigs inoculated with sputum remained well. The general condition of the patient was always good, and there was a steady gain in weight. Upon discharge from the Tuberculosis Hospital, Dr. Weber further recorded that there was grave doubt of the diagnosis of pulmonary tuberculosis, but no other disease process suggested itself.

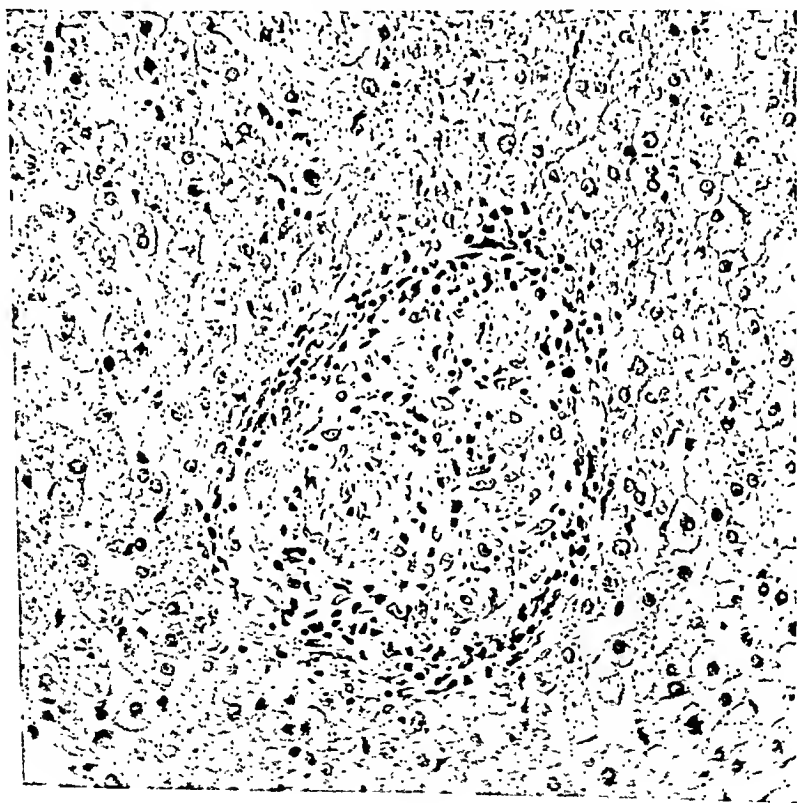


Fig. 5.

About the time the patient left this hospital, enlargement of lymph nodes over the body began and they increased in size rapidly. This lymphadenopathy was accompanied by the loss of 30 pounds in weight. One node was removed from the inguinal region on October 16, 1935, measuring 10 cm. in its greatest dimension. Enlarged glands were also present in the neck, postauricular and epitrochlear regions, and in the axillae. Histopathologic studies revealed lesions which resembled tubercles of the so-called "hard" variety in which no acid-fast bacilli could be found after extensive search. In spite of this failure to find the organisms a diagnosis of tuberculous lymphadenitis was submitted.

Because of the loss of weight, glandular enlargement, and a histologic diagnosis of tuberculous lymphadenopathy, the patient was kept in the out-patient clinic at the Tuberculosis Hospital.

Early in January, 1936, acute abdominal pain and distress began and increased in severity over a period of four weeks, after which time a second laparotomy was done at the Kingston Hospital. The liver this time was found to be far larger than upon the previous laparotomy, and its edge extended 10 to 12 cm. below the costal margin. It was studded with innumerable grayish-white nodules, most of which were only a few millimeters in diameter. Great enlargement of the perigastric lymph nodes was also seen. One of these lymph nodes and a small piece of liver containing one of the small nodules were removed for microscopic examination. Again the histologic diagnosis was tuberculosis involving the liver (Fig. 5) and perigastric nodes (Fig. 6), even though no acid-fast bacilli could be found in the lesions and macerated tissue injected into guinea pigs produced no disease.



Fig. 6.

Following this last operation and without specific therapy of any sort, the patient began to improve rapidly. During the period from February 16, 1936, to August 16, 1940, the clinical manifestations of the disease practically disappeared. He has been working for almost five years at his regular occupation as day laborer and feels entirely well. Early in August, 1940, inoculations of old tuberculin were done intracutaneously for the first time, in dilutions varying from 1:100,000 to 1:100, and yielded questionable reactions only in the lowest dilution. In June, 1941, small lymph nodes, barely palpable, were the only residual manifestation of the disease, while roentgenograms revealed complete resolution of the pulmonary and mediastinal changes previously observed, and no changes in the bones of the hands and feet could be demonstrated. Chemical examinations of the blood showed normal levels for calcium, cholesterol, total protein, and an albumin-globulin ratio of 2.75. No Bence-Jones protein was ever found in the urine.



## COMMENT

Although Hutchinson, Besnier, and Boeck described this malady as a dermatologic affection, it became evident to subsequent investigators that the cutaneous manifestations were merely part of a diffuse systemic disease of a granulomatous nature. Moreover, it is now clear that this skin eruption not only may not be prominent but often does not appear at all. Schaumann<sup>11</sup> in 1914 mentioned the possibility of the disease pursuing a concealed existence in the lymphatic and internal organs without giving rise to skin lesions, and that in all probability the skin was attacked in comparatively rare instances. Our cases demonstrate a predilection for the lymphatic system without skin manifestations and afford additional evidence of the validity of Schaumann's original assumption.

In the light of present knowledge it is difficult to accept our first two cases as instances of isolated glandular involvement. With the limited clinical means of exploring the body, obscure involvement of other lymphatic structures might well have been present. Although a thorough clinical examination of the peripheral glands and radiographic studies of the chest revealed no evidence of adenopathy, it has been pointed out that histologic examination of the clinically normal appearing lymphatic structures will often reveal sarcoids. Thus in Case 1, only after re-examination of a series of sections of tonsils removed five years previously was a single milium sarcoid demonstrated. Unfortunately, since no further studies were permitted, we were unable to observe the histology of nonpalpable glands or tonsils of Case 2.

The third case presented many signs and symptoms of a disseminated tuberculous process. There were slight fever with cough, weight loss, enlargement of the cervical, axillary, inguinal, mediastinal, and mesenteric lymph nodes, as well as pulmonary parenchymal infiltration of a caseous pneumonic type, and marked enlargement of the hilar shadows. Lesions in the liver and perigastric lymph nodes were found which resembled tubercles of the "hard" variety. Skin manifestations were absent, the tuberculin reactions were not significant, and tubercle bacilli could not be found in the sputum or excised tissues. The subsequent clinical course proved the diagnosis of milium tuberculosis to be incorrect, and re-examination of the extirpated glands established the true diagnosis. Since generalized Boeck's sarcoidosis is usually a benign, nonfatal disease, there is no doubt that many reported cures of milium tuberculosis are in fact instances of recovered sarcoidosis.

In this connection it is recalled with a great deal of interest by one of us how Dr. William S. Thayer at one time was thoroughly convinced that he had observed a case of milium tuberculosis which eventually recovered. A biopsy of the skin in his case revealed lesions which he and the pathologist interpreted as tubercles, but in which no acid-fast bacilli could be found. He was never quite satisfied that exhaustive search might not have revealed the presence of tubercle bacilli, and often regretted that animal inoculation had been rendered useless because of the fixation of the specimen before it reached the pathologic laboratory.

The true nature of the lesions has not been appreciated by many trained observers even though numerous descriptions of the histologic pathology have been recorded. Boeck mentioned sharply circumscribed foci of "new growth" composed of epithelioid connective tissue cells with giant cells of "sarcomatous" nature. Schaumann<sup>12</sup> in 1924 characterized the lesions as sharply circumscribed epithelioid tubercles without necrosis and very little lymphoid reaction. Nickerson<sup>13</sup> believes the following findings are of assistance in differentiating sarcoid from tuberculosis: (1) Caseation is never present. (2) The giant cells are different from those seen in tuberculous. (3) Lesions in tissues other than the lungs do not contain carbon pigment. (4) In the liver the lesions are most numerous in the portal triads, with only a few in the mid-zones of the lobules.

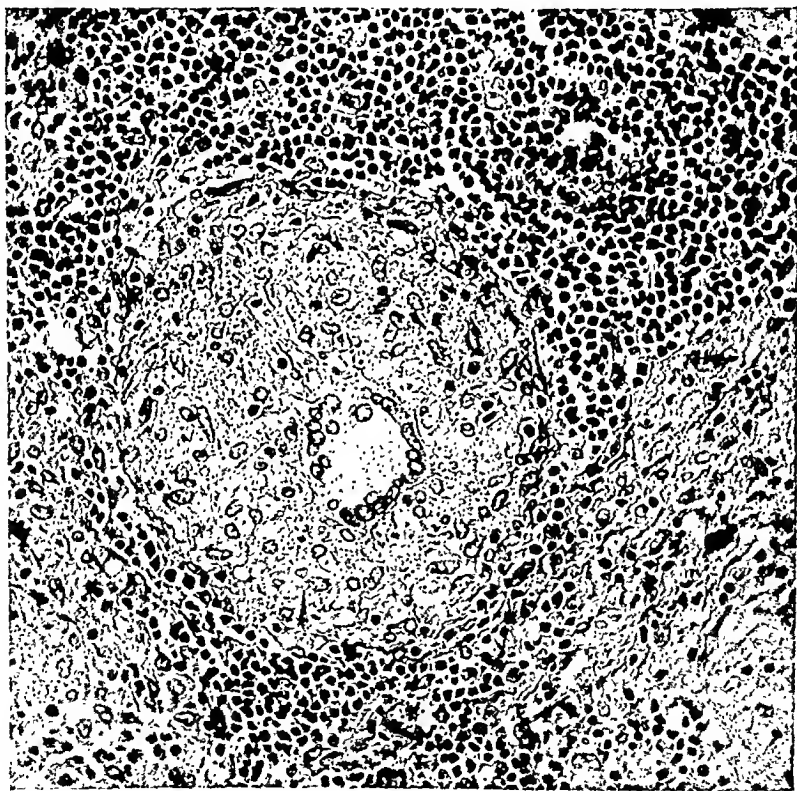


Fig. 7.

In miliary tuberculous the reverse is true. (5) With silver impregnation, delicate reticulum is always present in the lesion. In tuberculosis this is destroyed by the onset of caseation.

In our experience we have found it exceedingly difficult to differentiate sarcoidosis from truly noncaseating tuberculosis which even to the present time are not considered separate entities by some careful students of tuberculosis (Pinner<sup>14</sup>). However, recently our studies on the cases herein reported have convinced us that sarcoidosis is not an unusual form of tuberculosis but rather a distinct disease entity whose etiology is still obscure. The reasoning of Pinner

is interesting but not convincing. Often the problem confronting the pathologist is impossible of solution by mere histologic examination, yet there is a pattern presented by the sarcoid lesions to one who is familiar with them that enables him to distinguish the sarcoid from the noncaseating tubercle. Every student of sarcoidosis has recognized the absence of caseation and the slight peripheral lymphocytic reaction about the sarcoids, yet in a given true noncaseating tubercle a similar situation often obtains. Each attempt thus far to point out the distinguishing features between the sarcoid and the noncaseating tubercle has failed to convey in words what may readily be seen in sections. Our attempt perhaps also is futile, but it seems to us that the epithelioid cells in the individual sarcoid are usually larger and more cuboidal and that the individual sarcoids do not fuse to produce a conglomerate sarcoid in the manner that primary tubercles produce the conglomerate tubercle. The size of the giant cell, as well as the position and number of their nuclei, throw little light on the diagnosis. It can be seen in Fig. 7 that typical Langhan's giant cells may be present.

We urge those pathologists who do not yet recognize these sarcoid lesions to study all their cases of noncaseating tuberculosis with this disease in mind. Doubtless many clinicians, as well as pathologists, will need to observe a recovered case of "miliary tuberculosis," as was true with us, before they are convinced that the clinical-histologic picture is not that of tuberculosis but instead that of sarcoidosis.

We acknowledge our indebtedness to Doctors Fred S. Carr, Douw S. Meyers, and Frank A. Johnston for the privilege of studying these cases with them. The clinical observations herein recorded are essentially those made by these respective physicians. The photomicrographs were made by Mr. Lionel B. Herrington.

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# CLINICAL CHEMISTRY

## CLINICAL STUDIES WITH THE AID OF RADIO-PHOSPHORUS\*

### V. EARLY EFFECTS OF SMALL AMOUNTS OF RADIO-PHOSPHORUS ON BLOOD CELL LEVELS, UPTAKE, AND EXCRETION

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IT IS KNOWN that radioactive phosphorus emits  $\beta$  rays. Its half life is 14.3 days. When radio-phosphorus is administered orally or intravenously it has been found to localize in certain tissues of the body, namely, bone, bone marrow, neoplastic and leucemic tissue.<sup>1</sup> For this reason, radio-phosphorus has been used as a therapeutic agent in the treatment of leucemia, polycythemia vera, and lymphosarcoma. Extensive studies concerning the early absorption and distribution in blood and the early excretion in urine of therapeutic doses of radio-phosphorus in leucemia and polycythemia vera and in normal persons have been carried out.<sup>2</sup> These investigations have established definite patterns of response in the three groups in question when moderate-sized therapeutic doses are used. No standardization by means of very small doses has heretofore been attempted nor have any studies, so far as we are aware, been directed to the early effects of radio-phosphorus on blood cell levels in any of the three groups.

It is known that x-rays even in small doses produce biological effects long before therapeutic responses can be observed.<sup>3</sup> Such effects occur within the first twenty-four hours and concern fluctuations in peripheral blood levels, of red and white cells, reticuloocytes, and platelets, and the relationships of cells in the differential count. It has been our purpose in the present study to determine whether internal radiation resulting from intravenous administration of radio-phosphorus in small doses elicits similar responses. We have been concerned also with the early absorption and distribution in the blood of such doses, and with the rate of excretion in the urine. Attention has been given to the influence of previous x-radiation on the early response to radio-phosphorus, and we have attempted to differentiate between the effect of inert phosphorus and its radioactive isotope. Finally, we have observed the spontaneous fluctuations of blood cell levels over a period of three hours in some of the cases here presented.

The dose chosen was approximately 500 microcuries. Assuming a total body weight of 60 to 65 kg. and an even distribution of the radioactive metabolite, there would be 0.006 to 0.009 microcuries  $P^{32}$  per gram of body weight. We have arbitrarily called this a "threshold dose" for two reasons: First, in order

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to distinguish it from tracer doses used in animal experiments<sup>4</sup>; and second, because the biological effects are transitory and thus differ from so-called therapeutic effects.

The subjects have included apparently healthy persons and patients with various types of leucemia and with polycythemia vera.

#### METHODS

Radioactive phosphorus was produced in the 60-inch cyclotron<sup>5</sup> by the bombardment of red phosphorus which was then transformed into isotonic  $\text{Na}_2\text{HPO}_4$  solution containing 15 mg. of the compound per cubic centimeter. Subjects came to the laboratory in a fasting condition. Prior to the injection a blood count was made. This was repeated hourly for the first three hours after injection and at twenty-four hours. Withdrawal of 15 to 20 c.c. of blood by venipuncture was done at hourly intervals for the first three hours after injection and at twenty-four hours for the normal persons and for some of the patients. In one group of cases urine was collected at hourly intervals during the first three hours and at twenty-four hours.

One group of three leucemia patients prior to receiving any radio-phosphorus were given intravenously 3 c.c. (45 mg.) of inert  $\text{Na}_2\text{HPO}_4$ . Counts were made on these patients prior to injection and at hourly intervals for the first three hours and at twenty-four hours. A second group of patients was observed over a period of three hours for spontaneous fluctuations in blood cell levels.

The counts included: hemoglobin by the Osgood-Sahli method; red and white cell counts; platelets and reticulocytes by the cover slip method, using 1 per cent brilliant cresyl blue in 3.8 per cent sodium citrate. Smears were stained by the Wright-Giemsa technique, and at least 200 cells were enumerated for each differential count. The blood withdrawn from the veins was heparinized, cooled, and centrifuged for twenty minutes at  $1,450 \times$  gravity to insure constant volume. The plasma was removed, the buffy coat was aspirated, suspended in Riger's solution, and centrifuged again for twenty minutes at  $1,450 \times$  gravity.<sup>2a</sup> Finally the red blood cells were aspirated.

Materials for radioactive assay were measured, dried at  $90^\circ \text{C}$ ., and assayed in a standard DuBridge electrometer and compared with a uranium standard. The amounts measured did not exceed 20 to 30 mg. per square centimeter, and, therefore, no correction for  $\beta$  ray absorption is necessary.

#### RESULTS

Spontaneous fluctuations were negligible for all aspects of the blood count for each of the three patients observed. These included one case of chronic myeloid and two cases of chronic lymphoid leucemia. The other results are summarized in Tables I, II, and III.

The effects of inert  $\text{Na}_2\text{HPO}_4$  on two patients with chronic lymphoid leucemia and one with chronic myeloid leucemia are shown in Table I. The case of F. B. was characterized by only very slight elevation of the total white blood cell count, but this was made up almost entirely of lymphoid cells many of which were immature forms. The platelet count was very low from the outset.

TABLE I  
CHANGES IN BLOOD CELL LEVELS FOLLOWING ADMINISTRATION OF INERT  $\text{Na}_2\text{HPO}_4$

| CASE  | HB. % |     |     |     |     | R.R.C. MILLIONS |     |     |     |     | W.B.C. THOUSANDS |       |       |       |       | PLATELETS THOUSANDS |       |       |       |       | RETICULOCYTES % |     |     |     |     |
|---|-------|-----|-----|-----|-----|-----------------|-----|-----|-----|-----|------------------|-------|-------|-------|-------|---------------------|-------|-------|-------|-------|-----------------|-----|-----|-----|-----|
|   | 1     | 2   | 3   | 24  | HR. | 0               | 1   | 2   | 3   | 24  | 0                | 1     | 2     | 3     | 24    | 0                   | 1     | 2     | 3     | 24    | 0               | 1   | 2   | 3   | 24  |
|   | HR.   | HR. | HR. | HR. |     | HR.             | HR. | HR. | HR. | HR. | HR.              | HR.   | HR.   | HR.   | HR.   | HR.                 | HR.   | HR.   | HR.   | HR.   | HR.             | HR. | HR. | HR. | HR. |
| Case F. B.<br>Lymphoid leu-<br>cemia <sup>4</sup> | 109   | 109 | 109 | 104 | -   | 5.7             | 5.7 | 5.1 | 5.4 | -   | 15.7             | 14.9  | 14.4  | 13.0  | -     | 45.6                | 45.6  | 40.8  | 64.8  | -     | 1.2             | 1.2 | 1.0 | 1.0 | -   |
| Case P. P.<br>Myeloid leuce-<br>mia <sup>10</sup> | 73    | 76  | 73  | 72  | 74  | 3.9             | 4.3 | -   | 3.7 | 3.9 | 118.6            | 90.0  | 134.0 | 124.2 | 120.0 | 128.0               | 340.0 | 970.0 | 840.0 | 871.0 | 0.8             | 0.6 | 0.6 | 0.6 | 0.6 |
| Case L. C.<br>Lymphoid leu-<br>cemia <sup>5</sup> | 95    | 95  | 89  | 82  | -   | 4.8             | 4.5 | 4.7 | 5.1 | -   | 113.1            | 102.4 | 113.5 | 102.5 | -     | 117.2               | 244.0 | 397.0 | 171.5 | -     | 1.0             | 1.2 | 1.2 | 1.0 | -   |

None of the elements of the blood count exhibited any significant change. The case of chronic myeloid leucemia, which had been treated with x-rays up to three weeks before admission, exhibited some lability of the white blood cell count, possibly attributable to x-rays.

Cases 1, 2, and 3 of Table II show the typical hematologic response to 500 microcuries of  $P^{32}$  in three healthy persons. In all these subjects the platelets show marked deviations from the original levels during the first three hours. The shapes of the curves are the same in all three cases. There is a deviation beyond physiologic limits in the red blood cell count and hemoglobin, and this is closely correlated with the response of the reticulocytes. The total white blood cell count in all these cases remained unaffected.

Cases 4 through 10 of Table II show hematologic responses in lymphoid and myeloid leucemia patients. There is a definite effect on all elements of the blood count in these cases with the exception of the total white blood cell count of Case 4. Here it will be noted that the initial level of the white blood cells was only slightly elevated above normal. The platelet count in this case, which was unaffected by inert  $Na_2HPO_4$ , shows a definite rise and fall similar to those of the other cases of this group. The early radiation effect on the reticulocytes is especially pronounced in Cases 7 and 9. In all cases the point at which the reticulocyte level is at maximum the platelets are at minimum.

Cases 11 through 13 of Table II show hematologic responses in polycythemia vera. The red blood cells and the hemoglobin are the elements most profoundly affected, although all elements show some lability. The deviation of the reticulocytes is downward. The platelet count is markedly changed, but there seems to be no pattern of platelet response characteristic for this condition.

Case 14 of Table II shows the hematologic response in one case of polycythemia vera with leucemoid reaction. It would appear from this case that the typical responses of leucemia and polycythemia patients are here combined, with lability of the red blood cells, white blood cells, and platelets, and the reticulocytes exhibiting an upward trend.

Cases 1, 2, and 3 of Table III illustrate rates of uptake in three normal persons. The maximum excretion is approximately the same in all cases and is reached in all within the first three hours. Case 2 exhibits a somewhat higher uptake by white blood cells than the other two cases in this group. This may well be correlated with the somewhat higher level of the total white blood cell count in this person.

Cases 4, 5, 7, 9, 10 of Table III illustrate rates of uptake in leucemia patients. In Case 5 the utilization of phosphorus by white blood cells is in keeping with the excessive white blood cell proliferation as opposed to Case 4 where the total white blood cell count was never higher than 20,000. Case 9 shows a strikingly high uptake of phosphorus by white blood cells, despite the relatively low total white blood cell count. This may be explained on the basis that the differential count in this case consistently showed 85 to 90 per cent myeloblasts. It has been shown by Marshak<sup>6</sup> that cells in the early stages of mitosis are the largest consumers of phosphorus. Case 4 with a relatively low total white blood





|  |     |     |     |     |     |     |      |      |      |       |       |       |       |       |         |       |         |         |         |     |      |     |     |
|--|-----|-----|-----|-----|-----|-----|------|------|------|-------|-------|-------|-------|-------|---------|-------|---------|---------|---------|-----|------|-----|-----|
| 9. O. H. Subacute myeloid leucemia Male 26 yr.   | 39  | 39  | 39  | 42  | 2.4 | 2.2 | 2.4  | 2.5  | 2.1  | 11.3  | 13.0  | 14.3  | 11.5  | 20.8  | 150.0   | 107.0 | 102.0   | 269.0   | 602.0   | 0.1 | 14.2 | -   | 1.6 |
| 10. P. P. Chronic myeloid leucemia Female 32 yr. Recent treatment with x-ray                       | 74  | 76  | 72  | 76  | 3.9 | 3.9 | 3.6  | 4.2  | 3.7  | 120.0 | 103.3 | 103.6 | 121.8 | 117.2 | 871.0   | 780.0 | 860.0   | 870.0   | 920.0   | 0.6 | 0.9  | 1.0 | 0.8 |
| 11. G. Polycythemia vera Male 37 yr.   | 128 | 128 | 122 | 125 | 6.4 | 6.9 | 7.0  | 6.8  | 6.8  | 8.2   | -     | 10.7  | 8.6   | 5.2   | 2,636.0 | 829.0 | 189.0   | 609.0   | 750.0   | 2.0 | 1.6  | 0.7 | 0.9 |
| 12. F. M. Polycythemia vera Male 59 yr.  | 116 | 117 | 115 | 115 | 7.9 | 8.9 | 10.2 | 10.8 | 10.1 | 19.4  | 20.6  | 18.5  | 23.1  | 28.4  | 908.0   | 826.0 | 1,034.0 | 1,200.0 | 737.0   | 0.5 | 0.3  | 0   | 0.3 |
| 13. H. W. Polycythemia vera Male 77 yr.  | 90  | 88  | 97  | 88  | 6.6 | 7.3 | 7.5  | 6.4  | 7.8  | 54.4  | 48.1  | 49.4  | 44.3  | 47.3  | 1,400.0 | 973.0 | 2,180.0 | 636.0   | 1,100.0 | 2.2 | 1.1  | 0.9 | 0   |
| 14. H. H. Polycythemia vera with leucemoid reaction Male 65 yr. Previous treatment P <sub>32</sub> | 114 | 113 | 115 | 119 | 7.5 | 6.8 | 7.8  | 6.7  | 7.6  | 32.7  | 33.6  | 38.2  | 33.2  | 34.0  | 301.2   | 227.0 | -       | 80.4    | 880.0   | 3.1 | -    | 1.5 | 0.9 |

UTILIZATION AND EXCRETION OF  $P^{32}$ 

| CASE   | WHOLE BLOOD      |      |                  |      |                  |      |                  |      | R.B.C.           |      |                  |      |                  |      |                  |      |                  |       |
|--|------------------|------|------------------|------|------------------|------|------------------|------|------------------|------|------------------|------|------------------|------|------------------|------|------------------|-------|
|  | 1 HR.            |      | 2 HR.            |      | 3 HR.            |      | 24 HR.           |      | 1 HR.            |      | 2 HR.            |      | 3 HR.            |      | 24 HR.           |      | 1 HR.            |       |
|  | $\mu C$<br>/c.c. | %    | $\mu C$<br>/c.c. | %    | $\mu C$<br>/c.c. | %    | $\mu C$<br>/c.c. | %    | $\mu C$<br>/c.c. | %    | $\mu C$<br>/c.c. | %    | $\mu C$<br>/c.c. | %    | $\mu C$<br>/c.c. | %    | $\mu C$<br>/c.c. | %     |
| 1. D. V. N. Normal<br>Male 46 yr.  | 0081             | 1610 | 0094             | 1380 | 0081             | 1610 | 0061             | 1220 | 0011             | 0221 | 0106             | 2120 | 0130             | 2780 | 0131             | 2620 | 0192             | 3510  |
| 2. R. M. Normal<br>Female 38 yr.   | 0094             | 1879 | 0090             | 1799 | 0089             | 1783 | 0212             | 4240 | 0108             | 2159 | 0134             | 2680 | 0146             | 2925 | 0119             | 2382 | 0318             | 6370  |
| 3. F. V. N. Normal<br>Female 20 yr.  | 0125             | 2501 | 0126             | 2521 | 0187             | 3730 | 0077             | 1541 | 0113             | 2262 | 0141             | 2821 | 0120             | 2401 | 0127             | 2539 | 0165             | 3302  |
| 4. F. B. Chronic<br>lymphoid<br>leucemia<br>Male 40 yr.  | 0084             | 1681 | 0072             | 1442 | 0095             | 1901 | 0043             | 0860 | 0088             | 1759 | 0106             | 2119 | 0099             | 1980 | 0059             | 1179 | 0316             | 6310  |
| 5. L. C. Chronic<br>lymphoid<br>leucemia<br>Male 52 yr.  | 0084             | 1621 | 0074             | 1432 | 0063             | 1219 | 0052             | 0985 | 0034             | 1621 | 0095             | 1800 | 0095             | 1800 | 0095             | 1800 | 0286             | 5502  |
| 7. A. W. Chronic<br>myeloid<br>leucemia<br>Female 12 yr.   | 00894            | 1891 | 00824            | 1742 | ..               | ..   | 00282            | 0597 | 0020             | 0423 | 0082             | 1736 | ..               | ..   | 0062             | 1310 | 0821             | 17390 |
| 9. O. H. Subacute<br>myeloid<br>leucemia<br>Male 26 yr.  | 0030             | 0636 | 0018             | 0381 | 0018             | 0381 | 0009             | 0191 | 0040             | 0884 | 0042             | 0889 | 0052             | 1010 | 0036             | 0764 | 1150             | 2440  |
| 10. P. P. Chronic<br>myeloid<br>leucemia<br>Female 32 yr.<br>Recent x-ray                                | 0087             | 1735 | 0077             | 1541 | 0052             | 1039 | 0052             | 1039 | 0095             | 1901 | 0105             | 2010 | 0107             | 2013 | 0104             | 2080 | 0097             | 1941  |
| 11. G. Polycythemia<br>vera<br>Male 37 yr.   | 0303             | 4061 | 0309             | 4102 | 0209             | 2799 | 0172             | 2298 | 0296             | 3940 | 0362             | 4803 | 0391             | 5202 | 0302             | 4061 | 0464             | 6180  |
| 14. H. H. Polycythemia<br>with leucoid<br>reaction<br>Male 65 yr.<br>Previous treatment<br>with $P^{32}$ | 0247             | 4300 | 0216             | 3839 | 0213             | 3780 | 0275             | 4700 | 0060             | 1172 | 0259             | 4699 | 0229             | 4063 | 0251             | 4449 | 0617             | 1064  |

cell count shows a high level of excretion at the first hour, while the excretion rate of Case 5 with over 100,000 cells (white) reaches its peak at the third hour. The excretion of Case 7 is considerably lower than that of the normal persons or of the other patients in this group. This would seem to be in accord with the progressive uptake of phosphorus by newly forming white cells.

Case 11 of Table III shows uptake in a case of polycythemia vera. The red blood cells here show the highest uptake of phosphorus during the twenty-four-hour period of any of the groups studied. The difference between this case and the normal person in this respect, however, is very much less than the difference demonstrated between polycythemia and leucemia. The work of Erf and Lawrence<sup>2b</sup> indicates that the uptake of phosphorus by red blood cells is the same in normal persons and polycythemia patients at the twenty-four-hour point. These authors extended their observations beyond the first day, and their results

III

PER CENT OF DOSE PER 100 C.C.

| W.B.C.                    |        |                           |       |                           |        | PLASMA                    |       |                           |       |                           |       |                           |       | URINE                     |        |                           |        |                           |        |                           |        |
|---------------------------|--------|---------------------------|-------|---------------------------|--------|---------------------------|-------|---------------------------|-------|---------------------------|-------|---------------------------|-------|---------------------------|--------|---------------------------|--------|---------------------------|--------|---------------------------|--------|
| 2 hr.                     |        | 3 hr.                     |       | 24 hr.                    |        | 1 hr.                     |       | 2 hr.                     |       | 3 hr.                     |       | 24 hr.                    |       | 1 hr.                     |        | 2 hr.                     |        | 3 hr.                     |        | 24 hr.                    |        |
| $\mu\text{C}/\text{c.c.}$ | %      | $\mu\text{C}/\text{c.c.}$ | %     | $\mu\text{C}/\text{c.c.}$ | %      | $\mu\text{C}/\text{c.c.}$ | %     | $\mu\text{C}/\text{c.c.}$ | %     | $\mu\text{C}/\text{c.c.}$ | %     | $\mu\text{C}/\text{c.c.}$ | %     | $\mu\text{C}/\text{c.c.}$ | %      | $\mu\text{C}/\text{c.c.}$ | %      | $\mu\text{C}/\text{c.c.}$ | %      | $\mu\text{C}/\text{c.c.}$ | %      |
| .0218                     | .4352  | .0134                     | .2681 | .0313                     | .6251  | .0694                     | .1850 | .0031                     | .1079 | .0621                     | .0420 | .0019                     | .0379 | .0612                     | 1 2210 | .7368                     | 14 73  | 1 022                     | 20.39  | .075                      | 1.500  |
| .0251                     | .5001  | .0051                     | .1019 | .0134                     | .2681  | .0094                     | .1850 | .0030                     | .0600 | .0026                     | .0521 | .0013                     | .0200 | .0250                     | 5000   | .8205                     | 16 41  | .1550                     | 3 102  | .1075                     | 2.150  |
| .0202                     | .4040  | .0206                     | .4104 | .0114                     | .2280  | .0110                     | .2220 | .0073                     | .1459 | .0012                     | .0311 | .0027                     | .0510 | .0000                     | 12 000 | .9010                     | 18.020 | .9500                     | 19.00  | .2000                     | 4 001  |
| .0211                     | .4220  | .0125                     | .2501 | .0127                     | .2539  | .0016                     | .0021 | .0035                     | .0700 | .0034                     | .0680 | .0012                     | .0239 | .0910                     | 1 8750 | .9100                     | 18 750 | 1 120                     | 22 390 | .0630                     | 1 2509 |
| .0212                     | .4021  | .0382                     | .7243 | .0461                     | .8748  | .0020                     | .0379 | .0074                     | .1432 | .00124                    | .0797 | .0010                     | .0190 | .0403                     | .7600  | .4240                     | 7.970  | .8440                     | 16.00  | .1060                     | 2.121  |
| .0583                     | 1.2310 | .....                     | ..... | .1292                     | 2 7350 | .0103                     | .2150 | .0143                     | .3001 | .....                     | ..... | .0032                     | .0670 | .....                     | .....  | .....                     | .....  | .....                     | .....  | .0353                     | .7400  |
| .0801                     | 1.700  | .0940                     | 2.040 | .0961                     | 2 041  | .0115                     | .2441 | .0230                     | .4850 | .0118                     | .2503 | .0047                     | .0598 | Not done                  |        |                           |        |                           |        |                           |        |
| .0087                     | .1735  | .0061                     | .1221 | .0230                     | .4550  | .0063                     | .1261 | .0418                     | .8360 | .0031                     | .0620 | .00146                    | .0291 | .0230                     | .4590  | .8350                     | 16.699 | .1460                     | 2 910  | .0170                     | .3381  |
| .0433                     | .5770  | .0238                     | .3181 | .0161                     | .2149  | .0204                     | .2721 | .0204                     | .2721 | .0091                     | .1211 | .00290                    | .0388 | .3290                     | 4 371  | .....                     | .....  | .1570                     | 2.099  | .3450                     | 4.600  |
| .0492                     | .8730  | .0601                     | 1 063 | .0661                     | 1 172  | .0165                     | .2930 | .....                     | ..... | .00851                    | .1511 | .0119                     | .2105 | .2110                     | 3.741  | .0340                     | .6049  | .1945                     | 3.4410 | .0365                     | .6490  |

showed an increased uptake by red blood cells in one case of polycythemia vera at the forty-eight-hour point. It appears that no real basis of comparison exists for these findings and those of the present study for the following reasons: One point only in the uptake curves, namely, the twenty-four-hour period, is common to both investigations; the doses of radio-phosphorus (and the amounts in milligrams of salt in which they were incorporated) used by Erf and Lawrence are different for the patients and for the normal persons and in both groups the doses are considerably larger than the "threshold amounts" used in this study; finally the total number of cases observed in both studies is too small to rule out the existence of individual differences in response. The possibility that the red blood cells in polycythemia vera utilize radio-phosphorus to a greater extent than do normal red blood cells, suggests that investigations similar to those here discussed might well be extended to a larger group of

polycythemia patients, in the hope of establishing conclusively the uptake pattern in this condition. In the case here presented the excretion rate is correlated with the uptake phenomena and the curve is, therefore, lower in degree and opposite in trend from the excretion curves of healthy persons and leucemia patients.

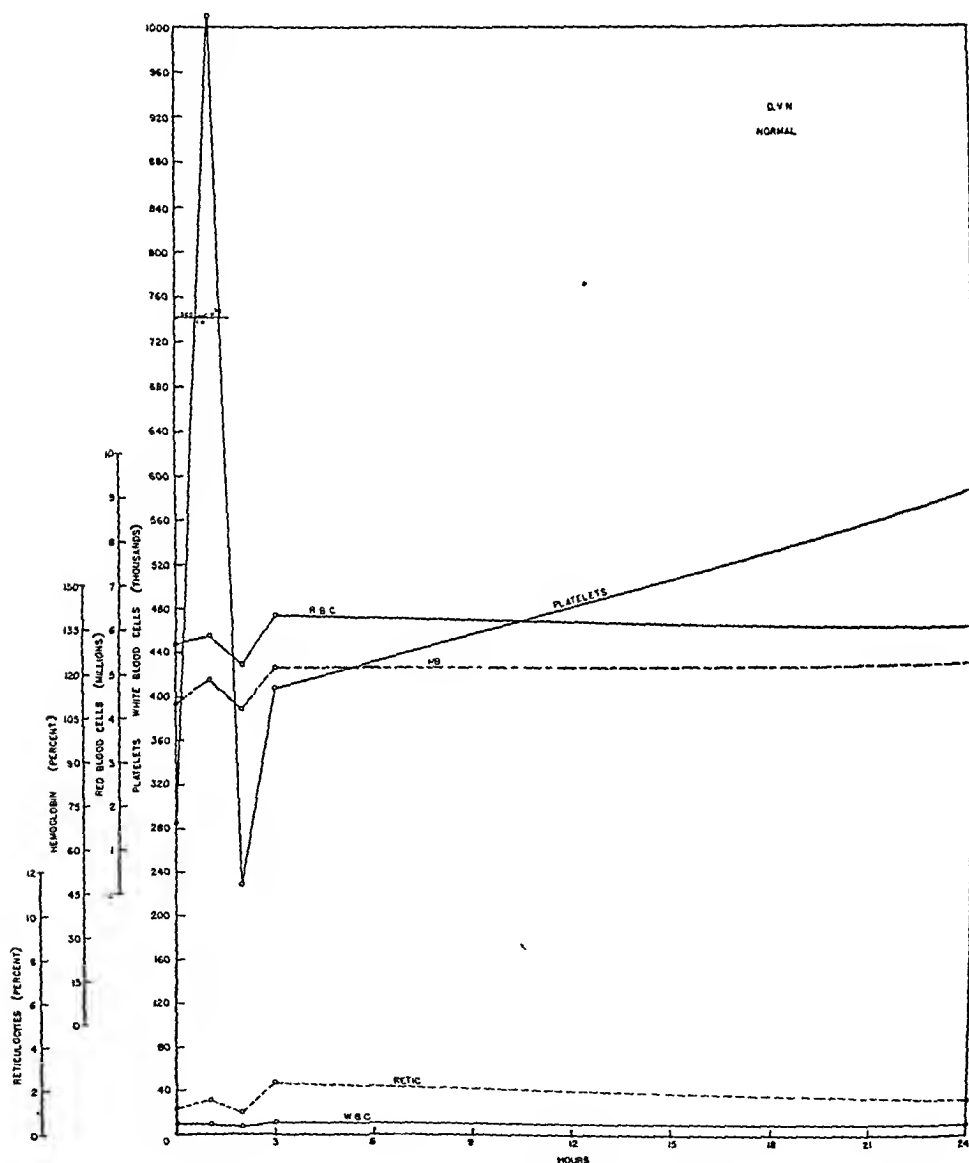


Fig. 1.—Hematologic response of normal persons to 500 microcuries  $P^{32}$  administered intravenously.

Case 14 of Table III shows uptake in the case of polycythemia with leucemoid reaction. The gradual increase in total activity of whole blood is in contrast to all other cases studied, and is no doubt accounted for by the high uptake of both red and white blood cells. The excretion curve follows those of the

leucemia patients in direction but differs in degree by reason of increased retention of radio-phosphorus by the blood.

With respect to the differential count, all the leucemia patients showed a slight increase in primitive cells (absolute numbers) during the twenty-four-hour period. This tendency did not appear in the polycythemia patients, although a few primitive cells were present in all these cases. The polycythemia patient with leucemoid reaction showed a decrease in primitive cells. There were

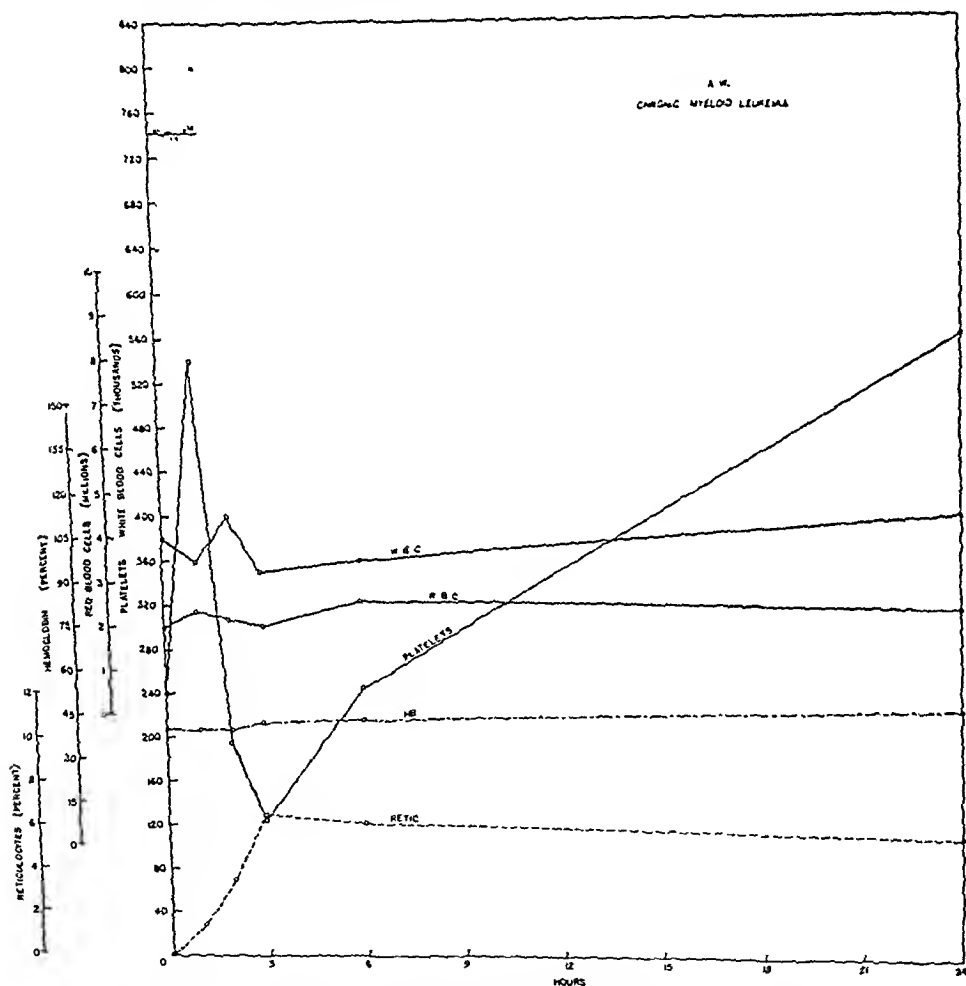


Fig. 2.—Typical early hematologic response of leukemia patients to 500 microcuries of  $P^{32}$  administered intravenously.

no shifts in the relationships of granulocytes and agranulocytes in any of the leucemia patients. The polycythemia patients showed a transitory increase in the relative number of lymphocytes during the first three hours.

The three normal persons showed no primitive cells at any time. Shifts in the relationships of other cells occurred, the most consistent being a transitory reduction in the absolute number of lymphocytes.

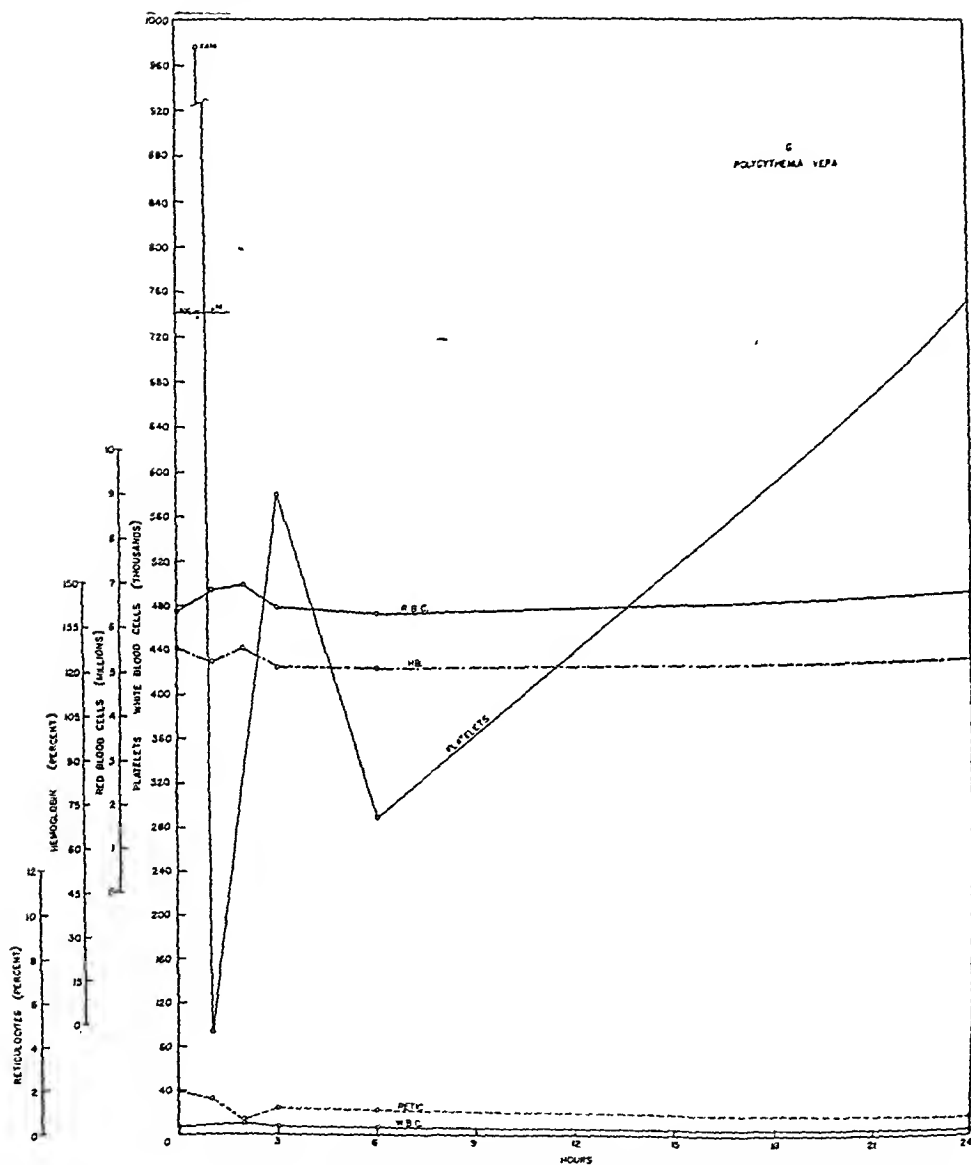


Fig. 3.—Typical early hematologic response of polycythemia vera patients to 500 microcuries of  $P^{32}$  administered intravenously.

#### DISCUSSION

The results show that radio-phosphorus in amounts of 0.006 to 0.009 microcuries per gram of body weight produces early radiation effects. These biological effects of very small activities of radio-phosphorus again indicate the importance of determining the "safe tracer dose" for each biological system studied.<sup>4</sup> The dose of radio-phosphorus used in these studies has been termed arbitrarily a "threshold dose." Experience in this study indicates that the actual threshold dose is individually conditioned by such factors as previous treatment and rate of cell production. Because of the known metabolic changes in leukemia and

polycythemia, knowledge of the basal metabolic rate might aid in predicting accurately a specific "threshold dose."

Group differences in uptake and excretion indicate the early function of the homeostatic mechanism in effecting an immediate distribution of  $P^{32}$  to the blood elements of highest metabolic rate. The excretion rate in normal persons is accordingly appreciably higher than in any of the patients.

#### SUMMARY

1. A threshold dose of radio-phosphorus has been arbitrarily defined.
2. Such a dose administered intravenously causes a definite "early radiation effect" on blood cell levels which is characteristic for each of the following groups: healthy persons, leucemia patients, and polycythemia patients.
3. This "early radiation effect" is further conditioned by the rate of cell production and by previous radiation treatment.
4. The uptake of  $P^{32}$  in the fractions of the blood and the rate of its excretion are roughly characteristic for each of the three groups.

We wish to express our appreciation to the staff of the Radiation Laboratory for their generous cooperation in providing the supply of radio-phosphorus used in this study.

The suggestions and criticisms of Dr. John H. Lawrence are gratefully acknowledged. One of us (B. V. A. L-B) wishes to express his appreciation for the privilege of taking part in this work at the Radiation Laboratory.

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## CHANGES IN THE CREATININE CLEARANCE AND URINE FLOW OF THE DOG DURING FEVER\*

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IN 1931 Goldring<sup>1</sup> reported that the urea clearances of human patients were usually higher in the acute febrile stage of rheumatic infection than the highest observed normal. He interpreted this as indicating a state of renal hyperfunction during the acute stage as a response to the demand of increased protein catabolism. In 1934 Grant and Medes<sup>2</sup> reported that the creatinine clearances of a large number of human patients with fever caused by infection were considerably higher when the body temperature was 38.9° C. or more, than when the temperature was between 36.7° and 37.1° C.

Grant and Medes<sup>2</sup> also reported that the creatinine clearance of normal unanesthetized dogs was increased after elevation of the body temperature by heating with conventional diathermy. But in the same year Page<sup>3</sup> reported that no significant change was observed in the urea clearances or in the urine flow of normal human subjects and patients with kidney disease during or after conventional diathermy. Farr and Moen<sup>4</sup> reported that the average urea clearance of rheumatic patients, placed in a cabinet heated by carbon filament lamps, with only the head and neck protruding, was 62 per cent of normal while the body temperature was rising and 75 per cent of normal when the body temperature was at a maximum of 104.8° to 106.7° F. Severe oliguria occurred in two experiments even though large quantities of water was given orally.

A new method of producing heat in tissues by high-frequency electromagnetic induction was introduced in 1934 by Merviman, Holmquest, and Osborne.<sup>5</sup> Mortimer and Osborne<sup>6</sup> found that during elevation of the body temperature of anesthetized dogs by this method, with the coil about the thorax, there existed a definite thermal gradient from the skin, which was heated to the highest temperature to the deeper tissues. The temperature of organs lying outside the short-wave field was elevated to a lesser degree. After heat induction was discontinued, the rectal temperature continued to rise for a short time while other parts of the body lost heat, showing that the heat was widely distributed by the circulation of the blood. Karr and Nassett<sup>7</sup> have elevated the body temperature of dogs, anesthetized with sodium amytal, to as much as 42.8° C. over a period of six hours by electromagnetic induction, during which time urine was collected by means of ureteral cannulae in hourly periods. They reported a decrease in total nitrogen excretion roughly parallel to the decrease in rate of urine

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flow, and found that oliguria and anuria were almost invariable sequelae to hyperthermia carried to  $42^{\circ}$  C. or higher. The blood pressure of their animals remained at a relatively high level until just prior to their death.

In 1938 Chasis, Ranges, Goldring, and Smith<sup>8</sup> reported that hyperemia of the kidneys occurred in human subjects during typhoid vaccine pyrexia. In the typical example they present, intravenous injection of 75 million organisms was followed in a few minutes by chills and a slight fall in body temperature, with a slight decrease in the inulin clearance. Within half an hour the inulin clearance increased about 10 per cent above normal and remained at that level, while the body temperature slowly increased over a period of six hours to a maximum of  $6^{\circ}$  F. above normal. Smith and his associates<sup>9</sup> have produced hyperemia of the kidneys of human beings by the intravenous injection of pyrogenic inulin. In the typical example presented, the body temperature began to increase slowly about an hour after administration of 80 mg. of pyrogenic inulin, and thirty minutes later the renal blood flow increased about 40 per cent and the inulin clearance increased about 50 per cent. The renal blood flow remained 40 to 50 per cent above the control level, and the inulin clearance varied between 10 and 50 per cent above the control level, while the body temperature increased slowly to a maximum of  $4^{\circ}$  above normal in a period of three hours.

This paper is a report of the effect of body temperature elevation upon the plasma creatinine clearance in unanesthetized dogs. The hyperthermia was produced by high-frequency electromagnetic induction, or by conventional diathermy, or by the intravenous injection of typhoid-paratyphoid vaccine, or by exposure to a current of cold air in a cold room.

#### EXPERIMENTAL PROCEDURE

Female dogs, trained to lie quietly during catheterization, vascular puncture, and heating, were used in the postabsorptive condition, sixteen to twenty-four hours after the last meal. Water was given by stomach tube in three or four doses before the first collection period in order to produce a urine flow of 2 to 5 c.c. per minute, and enough creatinine was given orally in order that the plasma creatinine should seldom fall below 10 mg. per 100 c.c. The bladder was washed twice with 20 c.c. of sterile 1 per cent sodium chloride solution before the first and at the end of each collection period. Approximately in the middle of each period enough blood was drawn to yield 1 c.c. of plasma for analysis. The plasma proteins were precipitated by means of the acid cadmium sulfate method of Miller and Van Slyke.<sup>10</sup> Creatinine was determined in the appropriately diluted urines and plasma filtrates by adaptation of the alkaline picrate colorimetric method<sup>11</sup> to the Evelyn photoelectric colorimeter,<sup>12</sup> with the following procedure:

Four cubic centimeters of a solution containing between 1.600 and 0.080 mg. per 100 c.c. of creatinine are pipetted into a dry photoelectric colorimeter tube. Into another such tube are put 4 c.c. of water, to be used as a blank. With each are mixed 2 c.c. of alkaline picrate reagent. After fifteen minutes the galvanometer is adjusted to read 100 with the tube of blank in the photoelectric colorimeter, using the No. 520 color filter and the No. 6 diaphragm. The blank is then replaced by the tube of unknown and the new galvanometer reading is recorded. From a table of known concentrations of creatinine and corresponding galvanometer readings, the creatinine content of the unknown is found.

Rectal temperature was measured at frequent intervals with a standard clinical thermometer. In these experiments fever is reported in degrees and tenths of a degree of temperature Fahrenheit above the rectal temperature of the dog during the control clearance periods.

High-frequency electromagnetic induction was accomplished with a Burdick SWD-10 Triplex. The electric current, with a frequency of 12 million cycles per second, was conducted through a flexible, heavily-insulated cable, supported by hard rubber clips in the form of a helix of three turns about the part of the body to be heated, from which it was separated by additional insulation of four layers of bath toweling. When heat was induced in the head of a dog, the helix was just posterior to the eyes and about the posterior part of the lower mandible. When heat was induced in the region of the kidneys, the helix was obliquely about the caudal three or four thoracic vertebrae and the anterior abdominal region. When heat was induced in the region of the pelvis, the helix was obliquely about the sacral vertebrae and the middle abdominal region. Two or three control clearance periods preceded a twenty-minute period of heat induction, immediately after which the cable and toweling were removed from the dog to allow the body temperature to return to normal, during which time there were one to six clearance periods. The controls of the apparatus were set to give approximately the same power output in each experiment. In two experiments a fever of approximately three hours' duration was maintained by intermittent high-frequency electromagnetic induction, in which ten-minute periods with induction were alternated with ten-minute periods without induction. In three dogs the lumbar sympathetic chains and the first sacral ganglia were excised and the splanchnic nerves were sectioned in two operations upon each dog. In two other dogs the kidneys only were denervated. When the wounds had healed, heat was induced for twenty minutes in the kidney region in two, three, or four experiments with each dog. In two electromagnetic induction experiments upon a normal dog and in one experiment upon a dog with lumbar sympathectomy, the diodrast clearance was used as a measure of renal plasma flow.<sup>13</sup> The analytical technique of White and Rolf<sup>14</sup> was used in the determination of diodrast iodine in urines and plasma filtrates.

Conventional diathermy was accomplished with equipment which included a Victor spark-gap diathermy machine, made by the General Electric X-ray Corporation, and a pair of pad electrodes, each of which was a thin sheet of lead,  $4\frac{1}{2}$  by  $8\frac{1}{2}$  inches in size, covered by several layers of surgical gauze. An electrode was held tightly against each of the shaved sides of a dog between the foreleg and the flank with a pad of toweling, about 1 inch thick 6 inches wide and 12 inches long, with several strips of water-proof adhesive tape completely encircling the pads and body of the animal. The electrodes and pads were kept wet with a 1 per cent sodium chloride solution, and a high-frequency alternating current of 1,500 milliamperes was allowed to flow through the body of the dog for one to three hours. There were clearance periods before, during, and after diathermy.

After two or three control clearance periods typhoid vaccine pyrexia was produced by the intravenous injection of doses of typhoid-paratyphoid vaccine

containing from 25 million to 2 billion killed organisms. The vaccine was obtained from the Wisconsin State Laboratory of Hygiene.

Two experiments were made with a dog, in each of which, after three control clearance periods in the moderately warm laboratory, the carriage supporting the animal was wheeled into a cold room and a current of cold air was blown against the shaved sides and abdomen by means of a large electric fan. After about one hundred minutes of such exposure the carriage was wheeled back into the laboratory and clearance periods were continued for about one hundred more minutes.

### RESULTS

High-frequency electromagnetic induction in any one of three body regions caused a rapid and continuous elevation of the rectal temperature, which was accompanied by vigorous panting. In a series of 18 comparative experiments with three dogs the average maximum hyperthermia observed at the end of twenty minutes was  $1.0^{\circ}$  F. when heat was induced in the head,  $1.9^{\circ}$  F. when heat was induced in the kidney region, and  $3.1^{\circ}$  F. when heat was induced in the pelvis. The temperature returned to normal in twenty to seventy minutes, and panting ceased at normal body temperature. The changes in minute volume of urine flow were inversely related to the changes in rectal temperature, as shown in Fig. 1. During maximum hyperthermia there was often an oliguria, but several minutes after the body temperature began to decrease, the rate of urine flow began to increase, until it was again between 2 and 5 c.c. per minute at normal temperature. There was no consistent relationship between the region in which heat was induced and the amount of urine flow decrease.

During the hyperthermia produced by electromagnetic induction, the plasma creatinine clearance generally decreased, but never significantly increased. In the series of 18 comparative experiments with three dogs the average maximum decrease in clearance was 13 per cent when heat was induced in the head, 45 per cent when heat was induced in the kidney region, and 27 per cent when heat was induced in the pelvis. A typical example of the results in each variety of these experiments is shown in Fig. 1, parts A, B, and C. When heat was induced in the kidney region in 28 experiments with nine dogs, a maximum decrease of the creatinine clearance of 12 to 79 per cent occurred twenty-two times, with a maximum hyperthermia of  $0.5^{\circ}$  to  $2.6^{\circ}$  F. In 12 of these experiments the clearance immediately increased to the control level as soon as induction was discontinued, even though the hyperthermia persisted. In eight of these experiments the clearance began to increase toward the control level as the body temperature began to decrease. In two experiments the clearance remained low. The eight experiments with no decrease in clearance during hyperthermia were with three large dogs in which it seemed impossible to produce a decrease in clearance except occasionally when the control clearances were high, i.e., more than 66 c.c. per minute.

In two experiments during electromagnetic induction of heat the plasma creatinine clearance decreased 44 and 32 per cent while the renal plasma flow decreased 32 and 35 per cent, respectively. The filtration fraction did not change significantly in either experiment. Immediately after induction was discon-

tinned, the creatinine clearance and the renal plasma flow increased again to the control values in both experiments.

The effect of electromagnetic induction after section of the splanchnic nerves and removal of the lumbar sympathetic ganglia is shown in Table I. The magnitude of decrease of the creatinine clearance during induction was much reduced as compared with that in the unoperated animal, or no decrease in the clearance occurred at all during induction after the operation.

TABLE I

EFFECT OF ELECTROMAGNETIC INDUCTION BEFORE AND AFTER SECTION OF SPLANCHNIC NERVES AND REMOVAL OF LUMBAR SYMPATHETIC GANGLIA

| DOG | BEFORE SYMPATHECTOMY |              |                         | AFTER SYMPATHECTOMY  |              |                         |
|-----|----------------------|--------------|-------------------------|----------------------|--------------|-------------------------|
|     | CONTROL<br>CLEARANCE | FEVER<br>°F. | CLEARANCE<br>DECREASE % | CONTROL<br>CLEARANCE | FEVER<br>°F. | CLEARANCE<br>DECREASE % |
| S   | 75.0                 | 1.0          | -33                     | 60.5                 | 0.5          | -18                     |
|     | 48.2                 | 0.7          | -46                     | 57.7                 | 0.4          | -None                   |
|     | 53.5                 | 1.5          | -71                     | 56.3                 | 0.9          | -20                     |
|     | 44.5                 | 0.5          | -71                     | 58.5                 | 0.9          | -30                     |
| W   | 30.1                 | 1.1          | -66                     | 38.7                 | 1.4          | None                    |
|     | 32.7                 | 0.6          | -46                     | 38.2                 | 1.6          | None                    |
|     | 28.4                 | 1.3          | -35                     |                      |              |                         |
|     | 32.1                 | 1.2          | -46                     |                      |              |                         |
| T   | 50.8                 | 1.3          | -56                     | 63.7                 | 2.7          | None                    |
|     | 47.3                 | 2.2          | -79                     | 61.0                 | 2.7          | None                    |
|     |                      |              |                         | 62.7                 | 2.2          | None*                   |

\*In this experiment there was no change in the renal plasma flow during or after heating, and the filtration fraction did not change.

After denervation of the kidneys only of each of two dogs, electromagnetic induction could cause a decrease in the creatinine clearance, as shown in Table II. Apparently dog K was one of that type of dog in which a decrease in the creatinine clearance sometimes occurs during induction and sometimes does not.

TABLE II

EFFECT OF ELECTROMAGNETIC INDUCTION AFTER DENERVATION OF THE KIDNEYS ONLY

| DOG | CONTROL<br>CLEARANCE | FEVER<br>°F. | CLEARANCE<br>DECREASE % |
|-----|----------------------|--------------|-------------------------|
| SP  | 39.7                 | 3.6          | -49                     |
|     | 40.4                 |              | -52                     |
|     | 54.6                 |              | -82                     |
| K   | 70.0                 | 2.1          | None                    |
|     | 66.2                 |              | -24                     |
|     | 62.2                 | 3.6          | None                    |

In order to make the hyperthermia produced by electromagnetic induction comparable to that of conventional diathermy both in magnitude and duration, two dogs were heated by intermittent electromagnetic induction in the kidney region for three hours. The body temperature ranged from 0.8° to 1.3° F. during this period, and the plasma creatinine clearance varied between the control level and 66 per cent of the control level. There was no increase in the clearance.

The effects of conventional diathermy upon five dogs in eight experiments were somewhat variable, as shown in Table III. In the first place, it was difficult

to induce a fever in dogs S and P, because these animals presumably were able to liberate heat very rapidly by violent panting. The other three dogs panted only gently and intermittently during diathermy, but at least an hour was necessary to produce more than a degree Fahrenheit of fever in them. There was only

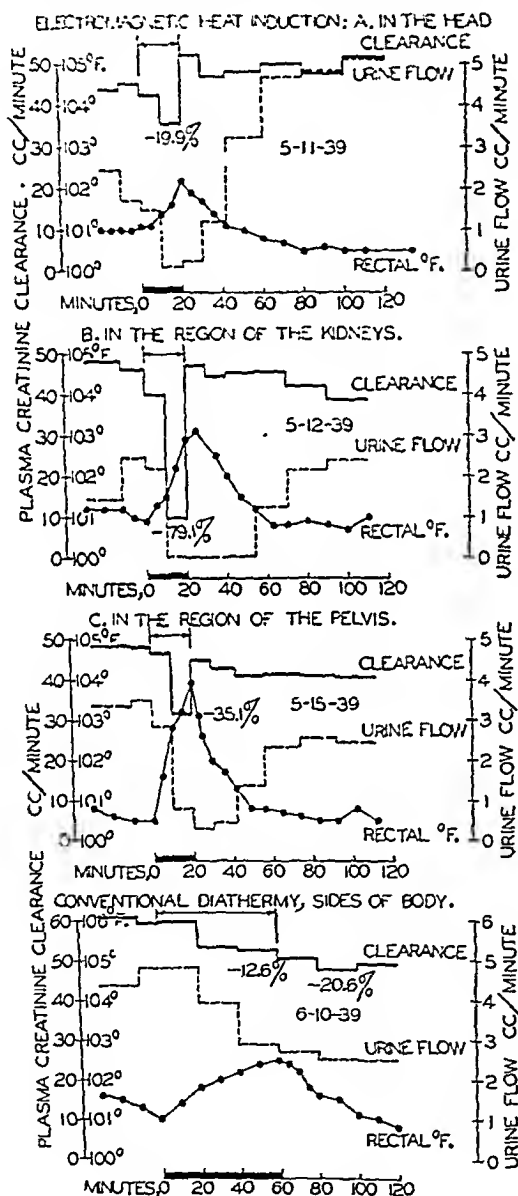


Fig. 1.—Typical changes in the plasma creatinine clearance and the minute volume of urine flow in a dog during fever produced by electromagnetic induction (A) in the head, (B) in the kidney region, and (C) in the region of the pelvis, and changes in clearance and urine flow in the same dog during fever produced by conventional diathermy.

a moderate, slow diminution of the minute volume of urine flow during the fever of conventional diathermy, as shown in Fig. 1. During diathermy there was either a decrease in the plasma creatinine clearance or no significant change,

except for a transitory increase of about 16 per cent in one experiment. After diathermy the clearance decreased still more in two experiments, decreased below normal in two others, and remained equal to or became equal to the controls in four.

TABLE III

PRINCIPAL CHANGES IN THE PLASMA CREATININE CLEARANCE OF DOGS DURING AND AFTER FEVER PRODUCED BY CONVENTIONAL DIATHERMY

| DOG | NORMAL               |              | HEATING PERIOD     |                          |              | AFTER HEATING            |              |
|-----|----------------------|--------------|--------------------|--------------------------|--------------|--------------------------|--------------|
|     | CONTROL<br>CLEARANCE | TEMP.<br>°F. | TIME IN<br>MINUTES | CHANGE IN<br>CLEARANCE % | FEVER<br>°F. | CHANGE IN<br>CLEARANCE % | FEVER<br>°F. |
| T   | 60.2                 | 101.0        | 60                 | -12.6                    | 1.5          | -20.6                    | 0.1          |
| R   | 63.3                 | 100.9        | 60                 | -15.8                    | 1.6          | -20.5                    | 1.1          |
|     |                      |              |                    |                          |              | To normal                | 0.0          |
| R   | 65.6                 | 100.6        | 49                 | None                     | 2.3          | -13.3                    | 1.4          |
| B   | 44.3                 | 100.5        | 59                 | None                     | 1.7          | None                     | 0.2          |
| S   | 63.8                 | 101.4        | 78                 | None                     | 0.0          | -16.1                    | 0.0          |
|     |                      |              |                    |                          |              | To normal                | 0.0          |
| S   | 49.0                 | 100.9        | 110                | +15.7                    | 0.5          |                          | 0.0          |
|     |                      |              |                    | To normal                | 0.9          | None                     |              |
| S   | 55.8                 | 101.2        | 180                | None                     | 0.6          | None                     | 0.0          |
| F   | 55.5                 | 100.2        | 180                | -11.0                    | 0.5          | To normal                | 0.1          |

The intravenous injection of typhoid-paratyphoid vaccine containing 25 million killed organisms into dog P was followed in a short time by a fever of 0.5° F., and later by an increase in the creatinine clearance of about 15 per cent. When a larger dose of 100 million killed organisms was used in each of twelve experiments with six dogs, there was no fever in two cases, but slight fever of 0.4° to 0.8° F. in ten; and no significant change in the creatinine clearance in six cases, but a moderate increase of 8 to 38 per cent in six, as shown in Table IV. In Experiments 12 and 13 an increase in the clearance was not observed until four and one-half to six hours after injection of the vaccine. When huge doses of 2 billion killed organisms were injected into dogs B and S, there was an immediate rise in rectal temperature to a maximum of more than 2° F. in about three hours. With subsequent huge doses of vaccine in dog S the onset of fever was later and the pyrexia was not so great as before. In Experiments 14, 15, and 16 the maximum clearance definitely did not coincide with the maximum fever but occurred earlier or later. In Experiments 15 and 17 the injection of the vaccine was immediately followed by an extreme, but transitory, decrease in the clearance. During all fevers by typhoid vaccine injection there was no panting and no decrease in the minute volume of urine flow other than that which normally follows a moderate diuresis.

It was found that exposure to a current of air from an electric fan in a room at 38° and 47° F. caused the dog to shiver violently and the rectal temperature to increase rapidly about a degree Fahrenheit above normal. This was followed by a decrease in the temperature to about half a degree above normal. In one experiment the plasma creatinine clearance increased to nearly 30 per cent above normal and in the other to 18.3 per cent. The minute volume of urine flow decreased only at a rate which normally follows a moderate diuresis. Upon return to the laboratory at 71° to 73° F., the rectal temperature fell to normal very quickly and the clearance decreased slowly toward the level of the controls.

TABLE IV

PRINCIPAL CHANGES IN THE PLASMA CREATININE CLEARANCE AND RECTAL TEMPERATURE OF DOGS AFTER INTRAVENOUS INJECTION OF TYPHOID-PARATYPHOID VACCINE

| EXPERIMENT NO. | DOG | NORMAL            |           | AFTER INJECTION OF KILLED ORGANISMS |                           |                            |                   |
|----------------|-----|-------------------|-----------|-------------------------------------|---------------------------|----------------------------|-------------------|
|                |     | CONTROL CLEARANCE | TEMP. °F. | DOSE IN MILLIONS                    | MIN. AFTER INJECTION      | CHANGE IN CLEARANCE %      | FEVER °F.         |
| 1              | P   | 65.8              | 101.5     | 25                                  | 62-83                     | +14.8                      | 0.5               |
| 2              | P   | 58.8              | 101.1     | 100                                 | 136-163                   | None                       | 0.5               |
| 12             | P   | 51.0              | 100.6     | 100                                 | 267-287                   | +38.2                      | 0.6               |
| 3              | F   | 57.2              | 100.5     | 100                                 | 0-171                     | None                       | None              |
| 4              | F   | 56.5              | 100.9     | 100                                 | 148-197                   | None                       | 0.4               |
| 13             | F   | 53.2              | 100.5     | 100                                 | 364-384                   | +14.3                      | 0.4               |
| 5              | T   | 43.6              | 101.0     | 100                                 | 0-175                     | None                       | None              |
| 6              | T   | 43.1              | 101.1     | 100                                 | 139-187                   | + 7.9                      | 0.8               |
| 7              | R   | 47.8              | 101.7     | 100                                 | 87-153                    | None                       | 0.4               |
| 8              | R   | 55.1              | 101.0     | 100                                 | 160-200                   | None                       | 0.8               |
| 9              | IV  | 55.7              | 100.6     | 100                                 | 145-177                   | +32.1                      | 1.0               |
| 10             | B   | 41.1              | 100.7     | 100                                 | 128-188                   | +25.3                      | 0.4               |
| 11             | B   | 40.6              | 100.1     | 100                                 | 153-201                   | +13.5                      | 0.6               |
| 14             | B   | 38.3              | 100.8     | 2000                                | 158-178<br>238-258        | +12.8<br>+23.5             | 2.4<br>2.1        |
| 15             | S   | 55.8              | 100.8     | 2000                                | 0-8<br>78-98<br>198-218   | -56.8<br>+28.1<br>+14.1    | 0.4<br>1.0<br>2.8 |
| 16             | S   | 48.8              | 101.0     | 2000                                | 221-241<br>371-391        | +32.0<br>+47.1             | 2.1<br>1.4        |
| 17             | S   | 62.6              | 101.1     | 2000                                | 0-10<br>88-108<br>258-278 | -69.0<br>To normal<br>None | 0.0<br>0.0<br>1.2 |

## DISCUSSION

The results obtained from these experiments suggest that the changes which occur in the creatinine clearance of the dog during fever or hyperthermia depend upon the causal agent. During hyperthermia produced by an external physical means, such as electromagnetic induction or conventional diathermy, there was generally a decrease in the clearance but never an increase, except in one instance when a transitory increase was followed by a decrease in the clearance. This is in agreement with the results of Farr and Moen,<sup>4</sup> who employed the external physical means of a heating cabinet, and also with the observation of Karr and Nassett<sup>7</sup> that heating with electromagnetic induction decreased the total nitrogen excretion. But these results are contrary to those obtained by Grant and Medes<sup>2</sup> with conventional diathermy. Inasmuch as the postabsorptive clearance of many dogs varies spontaneously from day to day, it is suggested that some of the increases in the creatinine clearance reported by Grant and Medes, which were above the control clearances determined on previous days, might have been found to be equal to or less than control clearances determined just previous to diathermy on the same day.

When fever was caused by vaccine injection or by exposure to cold, there was generally an increase in the clearance, but never a decrease, except in two

instances of transitory decreases followed by increases in the creatinine clearance to or above the control level. Both of these procedures may have produced hyperthermia by virtue of increased metabolic activity. A comparison of the results obtained with these four methods of producing hyperthermia leads to the conclusion that an increase in creatinine clearance was never due to hyperthermia itself, but rather to changes in the tissues which were responsible for the hyperthermia. As further evidence for this idea it may be noted that the maximum increase in clearance did not always coincide with the maximum body temperature, after injection of typhoid-paratyphoid vaccine. This is in agreement with the observations of Chasis, Ranges, Goldring, and Smith<sup>8</sup> with a human subject. Furthermore, Smith<sup>9</sup> shows that the inulin clearance of the human subject may increase about 50 per cent after injection of pyrogenic inulin long before the maximum body temperature is attained.

The decrease in creatinine clearance during hyperthermia produced by electromagnetic induction seems to have been due entirely to a reduction of the renal blood flow because of dilatation of blood vessels in the peripheral vascular bed. The fact that a decrease in the renal plasma flow during electromagnetic induction and an increase to the control level immediately after induction occurred simultaneously with the respective decrease and increase to the control level of the creatinine clearance, with no significant change in the filtration fraction, indicates that the decrease in renal blood flow was due either to constriction of the afferent arterioles or to extrarenal shunting of much of the blood to other parts of the body. These activities seem to be brought about in part or entirely by reflexes through the thoracolumbar outflow, because section of the splanchnic nerves and removal of the lumbar chains greatly reduced or entirely abolished the decrease in clearance and, according to one observation, the simultaneous reduction in renal blood flow. That the decrease in renal blood flow is not due to constriction of the afferent arterioles is indicated by the fact that denervation of the kidneys only did not prevent a decrease in the creatinine clearance during electromagnetic induction. The decrease in creatinine clearance during the hyperthermia produced by conventional diathermy may also have been due to a reduction of the renal blood flow because of dilatation of blood vessels in the peripheral vascular bed. It is suggested that the extreme, but transitory, decreases in clearance which occurred in the typhoid vaccine Experiments 15 and 17 of Table IV may have been due to a shunting of a large fraction of the blood from the renal arteries to the splanchnic vascular area because of a transitory shock accompanied by vasodilatation immediately following the injection of the huge dose of vaccine.

The inverse relationship between the minute volume of urine flow and the magnitude of the fever produced by electromagnetic induction was probably due to temporary water conservation and not to dehydration, because the urine flow increased soon after heating ceased. This change in the urine flow did not parallel the change in creatinine clearance throughout the duration of an experiment, nor did the amount of reduction in urine flow correspond to the amount of clearance decrease, as is shown in Fig. 1. Therefore, the regulation of the rate of urine flow seems to have been independent of the rate of glomerular



filtration. It is possible that secretion of the antidiuretic principle by the hypothalamus was increased as the body temperature was rapidly elevated, causing a proportional increase in the rate of water reabsorption in the kidney tubules. Attention is called to the fact that there was neither panting nor marked reduction in the rate of urine flow when fever was produced by typhoid vaccine injection. This may indicate a relationship between the mechanisms responsible for panting and for water conservation.

#### SUMMARY

During the hyperthermia produced by electromagnetic induction, the plasma creatinine clearance generally decreased, but never significantly increased. Heat induced over the kidney area was most effective in lowering the clearance, less effective when induced in the pelvis, and least effective when induced in the head. When heat was induced over the kidney area in 28 experiments with nine dogs, a maximum decrease of the creatinine clearance of 12 to 79 per cent, with a maximum hyperthermia of  $0.5^{\circ}$  to  $2.6^{\circ}$  F., occurred twenty-two times. This decrease of the clearance occurred about five minutes after the initial rise in temperature, but its magnitude was not related to the magnitude of the hyperthermia. The decrease of the clearance was found to be due to a simultaneous decrease of the renal plasma flow. Denervation of the kidneys only did not prevent a decrease of the clearance during the hyperthermia, but section of the splanchnic nerves and removal of the lumbar sympathetic ganglia nearly or completely prohibited this decrease of the plasma creatinine clearance.

An increase in body temperature of  $0.5^{\circ}$  to  $2.3^{\circ}$  F., caused by conventional diathermy, resulted in no significant change in the clearance in two experiments and a decrease of 11 to 21 per cent in five experiments. In one other experiment the clearance increased 16 per cent during the initial hyperthermia, but became normal again, even though the hyperthermia was maximum.

The intravenous injection of typhoid-paratyphoid vaccine resulted in a maximum fever of  $0.4^{\circ}$  to  $2.8^{\circ}$  in 15 experiments performed on seven dogs. In 10 of these experiments, an increase in clearance of 8 to 47 per cent occurred before, during, or after maximum hyperthermia. In five experiments there was no significant increase in the clearance. In only two of the 15 experiments did a decrease in clearance occur.

An increase in body temperature between  $1.0^{\circ}$  and  $0.5^{\circ}$  F. occurred during the exposure of a dog to a current of cold air at  $38^{\circ}$  and  $47^{\circ}$  F. in two experiments, during which the clearance increased 30 and 18 per cent, respectively.

Heating by the two electrical methods caused a reduction in the rate of urine flow irrespective of the region of heat production. On the other hand, the hyperthermias of typhoid vaccine injection and exposure to cold had no appreciable effect on the urine flow. A decrease in urine flow was due to an increase in the rate of tubular reabsorption of water and was independent of changes in the creatinine clearance.

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# LABORATORY METHODS

## GENERAL

### A STUDY OF THE PHENOMENON OF ERYTHROCYTE SEDIMENTATION\*

#### I. A CRITICAL SURVEY OF LITERATURE

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IN ANY sedimenting system there are two components which must be considered—the suspending medium (dispersing phase) and that which is suspended (the dispersed phase). Blood within the vessels may well be said to have the components necessary to classify it as a system capable of presenting the phenomenon herein referred to as sedimentation. It has a fluid suspending medium (the plasma) and suspended material (the formed elements). A certain degree of sedimentation can be demonstrated even within the vessels themselves if samples at various levels between two tourniquets are examined.<sup>73</sup> Before samples of blood drawn from apparently healthy horses can clot, considerable separation of cells and plasma occurs, much as is reported in samples from human beings during severe disease and from women during the latter stages of normal pregnancy.<sup>73</sup>

However, to measure the complete phenomenon of sedimentation of the formed elements of blood in their own plasma in vitro necessitates even in the horse, with its naturally very rapid rate of sedimentation, the use of artificial means of preventing clotting. The effects of this necessary alteration in the normal status of the blood upon the ultimate measurement of the phenomenon of sedimentation in vitro will be discussed later. Suffice to say that one should recognize that the determination of sedimentation in vitro represents not the phenomenon as it might occur in unaltered blood but rather as it occurs in blood necessarily changed to prevent clotting.

Despite recognition of the fact that a complete theoretical explanation of the phenomenon of sedimentation is probably impossible due to its marked complexity, mention of some of the various factors thought to be involved and the consensus of some of the experimenters concerning the relative value of these factors is included herein principally to convey the source of my conception of the sedimentation phenomenon. Due recognition is given the possibility that the opinions of some of these workers may have been acquired from results of techniques which have not measured the phenomenon completely.

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Reported data and opinions were segregated into six general groups: Physical Factors, Chemical Factors, Constancy, Physiological Factors, Technical Factors, and Pathological Factors. These were further subdivided where necessary. A conservative summary follows:

# I. PHYSICAL FACTORS

a. *Cells vs. Plasma*.—Both cells and plasma are apparently necessary elements in sedimentation. Changes in any of the physical and chemical properties of cells or of plasma may exert a variable influence upon the rate of sedimentation, the magnitude of this effect apparently being dependent upon not only the degree of alteration in any of these properties but also the properties altered.<sup>28, 48, 58, 62, 72, 73, 87, 96, 110, 111, 115, 138, 188, 213, 214, 217, 221, 227, 248, 253, 265, 321</sup>

b. *Specific Gravity*.—It would seem reasonable that for settling to take place the specific gravity of the formed elements must be greater than the specific gravity of the plasma. However, it also seems reasonable that the *degree of change* in the ratio of specific gravity of formed elements and plasma cannot by itself explain the magnitude of alterations in the sedimentation velocity which are encountered.<sup>24, 58, 72, 73, 115, 121, 149, 201, 214, 219, 225, 231, 301</sup>

c. *Viscosity*.—It may be said that ever-present viscosity of the plasma resists the settling of formed elements in plasma but that *changes* in the sedimentation rate cannot be explained wholly upon the basis of *changes* in viscosity.<sup>8, 17, 24, 25, 58, 67, 72, 73, 93, 115, 124, 130, 150, 187, 214, 225, 229, 231, 253, 255, 258, 263, 265, 301</sup>

d. *Movement of Plasma*.—The general movement of plasma is upward and tends to retard settling.<sup>72, 115, 150, 191, 241</sup>

e. *Particle Size*.—It would seem that the size of the individual cell has an important bearing upon its fall, particularly in suspensions where there is no rouleaux formation or agglutination. In suspensions where cells aggregate, variations in the size of the agglomerates of cells bear a definite relationship to the changes in sedimentation of the agglomerates—the larger the agglomerate the more rapid its fall.<sup>2, 22, 24, 31, 38, 40, 50, 52, 58, 72, 73, 115, 145, 150, 214, 220, 225, 227, 241, 242, 259, 263, 288, 301, 321, 344</sup>

f. *Number of Particles*.—Variations in the number of particles in a suspension produce variations in their rate of fall—the more present the slower each individual particle will fall. Since the number of cells present may influence, among other things, the number of aggregates, there probably exists a relationship between the number of aggregates and their rate of fall; with blood as a sedimenting system the more aggregates the slower their rate of fall.<sup>5, 18, 23-26, 29, 30, 33, 39, 48, 50-52, 58, 61, 67, 68, 72, 73, 76, 82, 89, 93, 96, 97, 99-102, 105-107, 109, 110, 113, 115, 125, 128, 136, 138, 142, 145, 150, 160, 170, 186, 190, 195, 203, 204, 211, 225, 227, 232, 235, 241, 242, 255, 257-259, 263-265, 272, 283, 288, 289, 318, 321, 322, 329-331, 333-335, 340, 341, 343, 344</sup>

g. *Rouleaux Formation and Agglutination*.—It seems universally accepted that the rate of sedimentation is considerably dependent upon rouleaux formation and subsequent agglutination—the larger the agglomerates of cells the more rapid their rate of fall.<sup>4, 17, 24, 28, 48, 58, 62, 63, 65, 72, 73, 82, 87, 93, 96, 115, 120, 125, 132, 141, 149, 150, 184, 188, 190, 194, 201, 214, 217, 221, 226, 227, 231, 236, 238, 241, 242, 253, 256, 258, 259, 263, 274, 280, 288, 289, 310, 321, 335, 338, 344, 348</sup>

*h. Environmental Temperature.*—It seems commonly agreed that decreases in the temperature of samples are accompanied by decreases in the rate of sedimentation, and that increases in temperature are associated with increases in the rate. It would appear that there is some difference of opinion as to the effects of heating plasma.<sup>5, 12, 51, 64, 72, 73, 79, 82, 93, 94, 96, 115, 145, 150, 156, 160, 161, 205, 211, 225, 227, 234, 241-243, 255, 258, 259, 282, 289, 300, 314, 321, 322, 329, 330, 340</sup>

*i. Other Physical Forces.*—Apparently such forces as the electrical charge of the cell, potential difference, cohesive forces, surface forces, repulsive forces, and osmotic pressure, exert influence either directly upon the sedimentation of agglomerates themselves or indirectly upon the phenomena of rouleaux formation and agglutination.<sup>5, 8, 24, 28, 41, 48, 70-73, 82, 87, 96, 129, 130, 132, 141, 149, 152, 157, 176, 179, 190, 182-184, 201, 203, 211, 217, 221, 234, 238, 242, 253, 258, 264, 265, 280, 285, 289, 312, 325, 326, 344</sup>

## II. CHEMICAL FACTORS

*a. Hemoglobin.*—That hemoglobin within normal ranges bears a fairly constant relationship to normal values of sedimentation is to be expected but correlation between *changes* in hemoglobin and *changes* in sedimentation is debatable.<sup>5, 22, 31, 39, 40, 45, 48, 50, 99, 100, 106, 111, 115, 138, 203, 212, 259, 263, 264, 283, 321, 329, 330, 340, 344</sup>

*b. Venous, Arterial, and Capillary Blood.*—The consensus seems to be that results from tests on venous, arterial, and capillary blood, as commonly obtained, agree.<sup>28, 54, 164, 204, 245, 258, 288</sup>

*c. Oxygen and Carbon Dioxide.*—While large variations in the oxygen or carbon dioxide content of blood experimentally may produce some alteration in sedimentation, simple aeration of blood prior to determination of sedimentation seems to be of no important significance.<sup>26, 73, 82, 100, 139, 155, 172, 204, 211, 234, 235, 245, 258, 260, 261, 327</sup>

*d. pH.*—There seems to be a difference of opinion as to whether variations in pH influence sedimentation even though some agreement exists that acidosis slows the rate and alkalosis hastens it.<sup>3, 5, 7, 8, 87, 156, 174, 201, 207, 228, 232, 234, 274, 344</sup>

*e. Protein Fractions.*—It would appear that increases in fibrinogen, and to a lesser extent perhaps globulin, have an accelerating effect upon sedimentation while increases in albumin have a retarding influence.<sup>1, 5, 8, 9, 12, 23, 24, 26, 28, 37, 43, 46, 48, 50, 51, 53, 57-59, 69-73, 78, 80, 82, 84, 87, 89, 93, 96, 97, 104-107, 115, 122, 128, 130, 132, 135, 138, 142, 145, 147, 152, 160, 163, 170, 173, 179, 180, 188-190, 196, 199, 201, 204, 206, 213, 214, 218, 227, 230-234, 236, 245, 246, 253, 254, 257, 258, 263, 265, 267, 268, 271, 273-275, 277, 282, 288, 290-292, 299, 305, 312, 325, 327, 328, 330, 333, 337, 342-344, 348</sup>

*f. Cholesterol and Lecithin.*—Increases in cholesterol are said to be accompanied by increases in sedimentation while increases in lecithin are said to be associated with decreases in sedimentation.<sup>44, 51, 58, 112, 115, 161, 162, 213, 232, 241, 247, 253, 254, 263, 274, 299, 320, 325</sup>

*g. Other Chemical Factors.*—That other chemical factors such as bile products, chlorides, lactic acid, lipid equilibrium and nitrogen balance, may influence sedimentation is not doubted, but whether any of these can offer a complete explanation of the phenomenon of sedimentation is assumably dubious.<sup>5, 8, 12, 25, 28, 39, 58, 96, 115, 130, 169, 182, 183, 234, 252, 254, 262, 265, 325, 344</sup>

## III. CONSTANCY

In the opinion of the majority a certain constancy of results during health apparently can be expected. However, there is enough opinion to the contrary to sug-

gest a careful critique of these techniques since a multiplicity of techniques have been employed.<sup>15, 33, 53, 64, 103, 115, 134, 145, 164, 190, 211, 232, 235, 274, 286, 288, 312, 321, 343, 344</sup>

The more literature that is reviewed the more one comes to realize that the phenomenon of sedimentation is very complex, and its measurement probably does not represent the measurement of any particular constituent or property or any limited group of constituents or properties of the blood sample being considered. It represents a measurement of a balance between, *on the one side*, the influence of *all* the constituents and properties, intrinsic or extrinsic, which augment the separation of the fluid and formed elements of a particular blood sample, altered so as to allow this separation to take place, and, *on the other side*, the influences of *all* the constituents and properties which delay this separation. If this conception be reasonable, then all constituents and properties, intrinsic and extrinsic, must be causal, even though variations in some may be of more relative importance than variations in others.

It would appear that alterations in the balance of these opposing constituents and properties may be the result of changes in the body itself (intrinsic) and changes brought about by the necessary manipulation of the blood sample (extrinsic or technical). Since the clinical value of the test probably lies in its use as a delicate measure of the intrinsic changes in the particular sample the result of disease, then the intrinsic changes of a normal physiologic nature and the extrinsic or technical factors which may produce changes in the manifestation of the phenomenon must be enumerated, evaluated, controlled or allowed for before the measurement of sedimentation can be said to reflect pathology.

#### IV. PHYSIOLOGICAL FACTORS

a. *Sex*.—The majority believe that the rate of sedimentation is faster in adult women than in adult men. There is some difference of opinion as to whether this difference can be accounted for solely by the difference in volume per cent of cells as noted between men and women.<sup>10, 53, 72, 73, 96, 103, 105, 106, 108, 137, 138, 202, 259, 264, 340, 343</sup>

b. *Pregnancy*.—Pregnancy is recognized as causing an increase in sedimentation, being first noted after the first few months, reaching its peak at parturition and gradually returning toward normal during puerperium.<sup>8, 11, 14, 27, 28, 48, 51, 57, 71-74, 82, 93, 96, 103, 127, 138, 180, 183, 184, 194, 201, 202, 211, 233, 250, 267, 270, 274, 283, 322, 330, 343</sup>

c. *Menstruation*.—Some authors find an increase in sedimentation during menstruation. Others doubt an increase above normal limits.<sup>53, 103, 138, 180, 181, 274, 295, 322, 340, 343</sup>

d. *Age*.—A difference of opinion exists regarding age but the majority find a slower sedimentation in adults than in young or old persons.<sup>53, 72, 73, 105, 138, 198, 202, 211, 225, 288, 301, 312, 343, 346</sup>

e. *Food*.—That ingestion of food has no effect upon sedimentation seems at least generally accepted.<sup>48, 61, 97, 105, 145, 211, 258</sup>

f. *Exercise*.—Again a difference of opinion exists. Probably the degree of exercise is important since enforced rest is reported as delaying sedimentation and overwork as increasing it.<sup>211, 225, 232, 258</sup>

g. *Sweating*.—Profuse sweating by electric baking had no effect on sedimentation.<sup>8</sup>

h. *Excitement*.—Tests taken at times of greater excitement in children gave higher readings.<sup>312</sup>

i. *Time of Day*.—A difference of opinion exists as to whether or not the time of day has any direct effect upon the sedimentation rate.<sup>134, 164, 211, 258</sup>

j. *Atmospheric Conditions*.—Atmospheric conditions are reported as having some effect on sedimentation.<sup>94, 134, 116, 225, 301, 312</sup>

#### V. TECHNICAL FACTORS

1. It would seem that *agreeable results* upon the same sample can be obtained with certain techniques. However, literature upon this subject is very deficient and techniques differ considerably.<sup>72, 115, 308, 310</sup>

2. It seems commonly accepted that *venous stasis* should at least be minimized, since if it is applied in excess, sedimentation is usually considerably retarded.<sup>82, 93, 145, 204, 211, 232, 231, 259, 264, 265, 288, 321, 330</sup>

3. Too little *anticoagulant*, as well as too much anticoagulant, is to be avoided. Anticoagulant and blood must be properly mixed. Different anticoagulants yield different results. Although solutions of sodium citrate have probably been used more than any other anticoagulant, the present tendency is to lean toward the anticoagulant mixture of *Heller and Paul*<sup>123</sup> and toward heparin, for these two seemingly produce little variation in cell volume as well as sedimentation. Differences of opinion naturally exist concerning the proper kind and amount of anticoagulant to use.<sup>8, 20, 23, 29, 32, 39, 50, 51, 58, 60, 64, 72, 76, 95, 101, 105, 109, 115, 119, 123, 125, 133, 140, 150, 216, 221, 224, 232, 235, 241, 245, 249, 257, 258, 264, 280, 288, 311, 321, 322, 329, 340, 341, 345</sup>

4. It would seem that the general consensus is that *delay* in conducting a measurement of the sedimentation of a sample may cause retardation of the rate. The maximum amount of permissible delay reported is variable from a few hours to several hours. It is suggested that the retarding effect of delay may bear some relationship to the kind of anticoagulant used and also the temperature at which the sample is kept.<sup>29, 51, 54, 60, 64, 72, 75, 93, 108, 115, 119, 136, 142, 154, 156, 164, 185, 186, 201, 211, 224, 227, 239, 240, 242, 258, 259, 281, 283, 288, 329, 330, 338, 340, 344, 348</sup>

5. Although it has been recognized that a *uniform mixture of cells and plasma* is necessary at the start of measurement of sedimentation, it would seem that little attempt has been made to demonstrate that such a uniformity is actually established.<sup>50, 55, 94, 115, 136, 154, 164, 185, 211, 258, 259, 308</sup>

6. There has been a multiplicity of *tubes* used for the measurement of sedimentation. However, there seem to be certain generalities which are quite commonly accepted. Capillary tubes are thought not to be dependable. The majority of workers used tubes of over 2 mm. bore and apparently thought them satisfactory. The general opinion seems to be that longer tubes yield faster rates, yet there is reason to doubt this upon a physical basis. It seems recognized that the sides of tubes should be parallel and the bottoms should be flat.<sup>8, 10, 12, 17-19, 26, 28, 29, 33, 36, 50, 51, 54, 55, 57, 58, 60, 64, 72, 76, 78, 79, 82, 94, 96, 101, 106, 108, 109, 114, 115, 118, 137, 145, 154, 164, 171, 180, 193, 198, 204, 211, 214, 225, 227, 234, 235, 241, 250, 258, 259, 263-265, 273, 278, 287, 288, 293, 315, 317, 321, 322, 325, 329, 330, 340, 341, 343, 348</sup>

7. The necessity of *perpendicularity of the tube* seems generally recognized.<sup>12, 17, 19, 29, 34, 36, 42, 64, 73, 79, 114, 115, 154, 164, 191, 193, 210, 211, 225, 227, 235, 241, 259, 264, 293, 317, 321, 330, 340, 348</sup>

8. It appears generally accepted that sedimentation takes place in three stages: (a) Initial aggregation and accelerated fall. (b) Constant fall. (c) Slowing or retarded fall.

There is evidence that sedimentation occurs as a sigmoid curve. This would render dubious the possibility of a phase of constant fall.<sup>17, 29, 33, 58, 64, 73, 115, 191, 215, 259</sup>

9. There exists a great variety of *methods of expressing sedimentation*, the great majority of which depend upon a relationship between the position of the line of demarcation between cells and plasma and the amount of time since the test was begun. Some have used the amount of time necessary for the border between cells and plasma to reach a certain mark, some have noted the position of this line at a given time, and others have expressed the descent of this border line by reading its position at definite time intervals. Many of the mentioned methods probably fail to express the phenomenon *completely* and some probably do not represent expression of *any part* of what is generally considered the most important phase of the phenomenon, namely, aggregation. Multiple readings of the degree of settling at frequent time intervals or mechanical means of measuring the rate seem to offer advantages.

It is felt that evidence from results with an automatic photographic device, which continuously records the rate of descent of the line of demarcation between cells and plasma,<sup>215</sup> is convincing that the best measurement of the phenomenon of sedimentation, as evidenced by the rate of descent of the line of demarcation between cells and plasma, is a continuous measurement with estimation of the point of change in the resulting sigmoid curve. Such estimation, if even partially to express the important aspects of the curve, must include the amount of separation before the point of change takes place, the time at which the point of change occurs, and the rate or velocity of separation immediately preceding the point of change (expressed as an angle between the steepest portion of the sigmoid curve and perpendicularity).<sup>7, 10, 12, 17-19, 23-25, 29, 30, 33, 36, 37, 50, 53, 55, 57-59, 61, 64, 68, 69, 72, 73, 76, 79, 88, 93, 94, 96, 99-101, 103, 105-108, 110, 113-115, 118, 119, 134, 136, 137, 150, 154, 159, 164, 171, 180, 181, 186, 190, 194, 195, 198, 201, 211, 214, 215, 218, 225, 227, 235, 241, 258, 259, 264, 265, 273, 278, 287-289, 293, 295, 298, 301, 302, 308, 315, 317, 321, 325, 328-331, 333, 334, 340</sup>

## VI. PATHOLOGICAL FACTORS

Inclusion of several references brings out the generally accepted theory that alteration of the sedimentation phenomenon portrays metabolic changes—the result of nonspecific destructive disease.<sup>5, 11, 28, 33, 47, 48, 51, 53, 55, 57, 72, 76, 78, 79, 87, 103, 110, 114, 115, 174, 195, 198, 201, 211, 259, 265, 274, 323, 330, 339, 343</sup>

The second of this series of articles will deal with the establishment of a reliable technique with evidence of its reproducibility and limitations.

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## THE EFFECT OF OPERATOR ON THE VARIABILITY OF BASAL METABOLISM DATA

DENA CEDERQUIST,\* MANHATTAN, KAN.

**S**TUDIES of basal metabolism reported in the literature suggest that all tests were made under standard conditions. Standard conditions for the determination of basal metabolism have come to mean that the person was in a postabsorptive state, comfortably warm, had a normal body temperature, was awake but at complete rest and free from the effects of physical exercise, mental and physical irritation, and fear. As it is almost always possible to meet these conditions, the error associated with technical procedure would be expected to be reduced to a minimum. Thus two successive observations made on one person under the foregoing conditions should agree closely, or in the language of the statistician, the two observations should show little intraday variation. However, in actual practice, more or less variation tends to occur.

In this laboratory 450 tests on 225 women between 15 and 45 years of age were made under what were believed to be standard conditions. A Benedict-Roth closed circuit machine was used. In most cases the subjects were brought to the laboratory by car and were required to rest in bed one-half to one hour before the observations were made. A few of the tests were made in a women's residence hall. For these, the metabolism apparatus was moved into the subject's room, and the observations were made in the morning before she arose. Two eight-minute tests with a three- to five-minute rest period between them were made on each person. All observations were used unless some obviously disturbing factor, as an unexpected noise, affected the test. Five technicians were responsible for collecting these data.

Because the observations were made under standard conditions using the same equipment, it would seem logical to expect that the variation observed in individual records made on the same day would be of the same magnitude for the entire group. To determine if this were true, an analysis of variance was made on the 450 tests with the records divided on the basis of the technician who made the test. That portion of the analysis relative to the variation observed in records made on the same day is shown in Table I.

In the interpretation of this portion of the analysis of variance, the size of the mean square is used as a measure of intraday variation. The larger the value of the mean square, the greater is the variation in the observations made on one person on any given day. It can be seen from Table I that this variation,

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as indicated by the value of the mean square, is not the same for all technicians. It will be noted that for Technician O the mean square of the records on the same day is about two and one-half times that of Technician B. This difference is great enough to be of statistical importance. It indicates that successive tests made by Technician O show significantly more variability than those made by Technician B. This observation has suggested that in this study, where the same piece of apparatus was used each time under what is believed to be standard conditions, some of the differences in the amount of intradaily variation may be attributed to the technician.

TABLE I  
ANALYSIS OF INTRADAILY VARIATION

| TECHNICIAN | DEGREES OF FREEDOM* | MEAN SQUARES† |
|------------|---------------------|---------------|
| B          | 62                  | 1.42          |
| C          | 232                 | 1.61          |
| R          | 42                  | 2.17          |
| K and C    | 124                 | 2.18          |
| O          | 48                  | 3.48          |

\*Degrees of freedom =  $(n-1)$  where  $n$  refers to the number of observations.

†Mean square or variance =  $\frac{\text{sum of the squares of deviation from mean}}{\text{degrees of freedom}}$

No definite explanation for the differences noted between technicians is offered, but a plausible suggestion is that they may be due to differences in sensitivity of the observer. For example, one technician might note every unexpected noise, draft, or bright light which could disturb her subject and would discard the test because it was not done under standard conditions. Another operator might not recognize such disturbing factors and would believe that standard conditions had been established when in reality they had not. Also, not all persons approach a given situation with the same degree of relaxation. If the operator is tense, it is possible to induce a tension in the subject without the latter realizing she has been disturbed. In either case a source of error has been introduced which will increase intradaily variation.

Part of the intradaily variability in basal metabolism tests on the same person may often be attributed, therefore, to some characteristic of the technician. When all technicians are carefully trained, the differences in variability cannot be attributed to careless workmanship, but may be accounted for by differences in sensitivity to the interpretation of standard conditions. This study suggests that, even though standard methods have been outlined for the determination of basal metabolism tests, it is possible for the technician to introduce variability and thus decrease the reliability of the tests.

## LABORATORY SHAKING MACHINE\*

E. E. MYERS, M.D., PHILIPPI, W. VA.

A STURDY, silent shaking machine, which can be easily built in a small machine shop, is shown in the photograph. The diagram gives detail of construction. The length and period of excursion are those specified for use in the Kahn test. With used connecting rods and a used motor the machine can be built for twenty-five to thirty dollars.

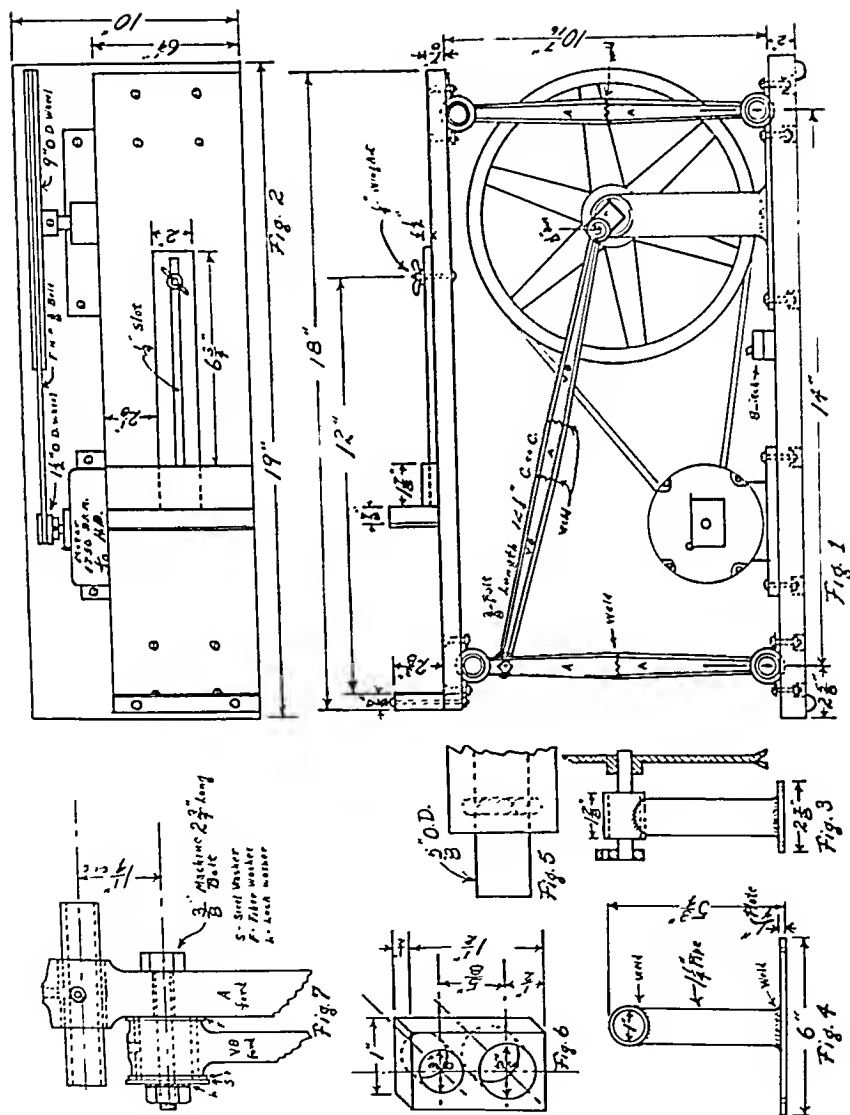
Fig. 1 in the diagram is a side view of the shaking machine. The rocking arms are model A Ford connecting rods with the crankshaft-bearing portions cut off. They are welded together to make an overall length of  $10\frac{7}{16}$  inches. The wrist pins must be parallel; this can be accomplished by a wooden jig that has parallel blocks on which the pins are held by clamps during the welding.

The wrist pins are clamped to the baseboard and top board by  $\frac{3}{4}$  inch pipe clamps, the boards being hollowed slightly to allow the connecting rod parts which extend beyond the wrist pins to have freedom of movement. Side motion is prevented by the lock rings in the connecting rods. The oil holes on the bottom pieces are plugged with wooden plugs. Celluloid cement can be coated over the ends of the plugs to prevent seepage of oil through the paint. The oil hole at the top is left open to receive oil (about number 10 motor oil is used for lubrication). All wrist pins should be a medium tight fit only.

The throw arm is made from two V-8 Ford connecting rods with a short piece of model A rod welded between them to give a total length of  $12\frac{1}{4}$  inches from center to center. The wrist pins of the throw arm are cut off just the width of the throw arm plus the thickness of two fiber washers which go one on either end of the pin. Fig. 7 shows another view of the connection of the throw arm to the rocking arm. The  $\frac{3}{8}$  inch bolt is drawn up tightly, holding the wrist pin securely against the surface of the rocking arm, which, where the pin strikes it, is filed to a flat surface. The fiber washers shown as *F* in Fig. 7 then act as spacers for the throw arm, eliminating noise. The connection of the throw arm to the eccentric is made in a similar manner. The plate for the eccentric is shown in Fig. 6. The holes in it must be parallel, and after it is welded to the shaft of the bearing shown in Fig. 5, the surface to which the throw arm is connected should be machined in a lathe to give a flat surface parallel to the throw arm. This is essential to prevent binding of the throw arm during rotation of the eccentric. The  $\frac{3}{8}$  inch machine bolts used to fasten the throw arm bearings are somewhat smaller than the holes in the wrist pins through which they pass, and this permits adjustment of the arm on the eccentric to produce the exact excursion of the top board of  $1\frac{1}{2}$  inches.

\*From the Laboratory of the Myers Clinic, Philippi.  
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**Figs. 1-7.**

The bearing illustrated in Figs. 3 and 5 is an automobile packless type water pump ball bearing and shaft, New Departure, No. 885156S, 108556. While welding the plate to the shaft of this bearing, care must be taken to prevent the bearing from becoming too hot, as by wrapping wet asbestos paper around it. Electric welding is necessary here. The bearing case is pressed into a piece of 1 inch pipe coupling which can be used without alteration, i.e., the threads are left intact. Before the bearing is fitted, the coupling is welded to a piece of  $1\frac{1}{4}$  inch pipe, as shown in Figs. 3 and 4. This pipe is welded to a plate which forms the base to be attached to the bottom board by four bolts.

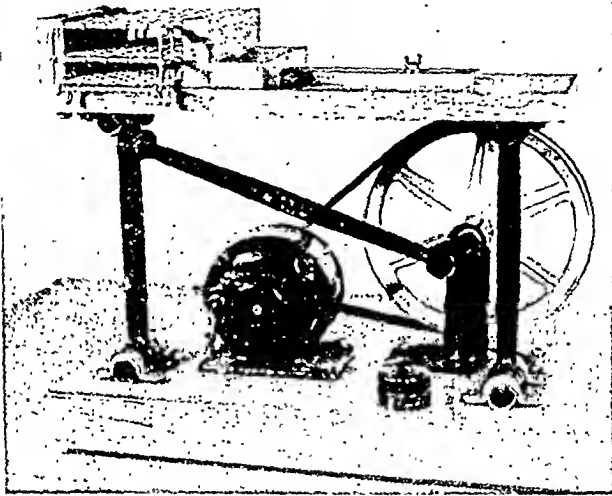


Fig. 3.

Fig. 8 is a top view of the completed machine. The boards forming the clamp for the Kahn racks are covered with rubber cut from stair tread. These rubber pieces can be cemented or tacked with small brads. Carriage bolts  $\frac{1}{4}$  by  $3\frac{1}{2}$  inches are used to attach the end board. A block with a V in it can be made for holding a bottle, permitting it to be clamped in place of a rack.

The motor is a 1,750 r.p.m. and  $\frac{1}{30}$  h.p. (or larger). Using a  $1\frac{1}{2}$  inch pulley on the motor, the speed will be slightly slower than that required. The speed can be increased by wrapping tire tape around the pulley in a direction opposite that of its rotation. The speed is checked after each addition of one or two layers of tape until the revolutions per minute of the large wheel, as determined by a speed indicator (or by oscillations of the top board), are between 275 and 285.

Diameters of pulleys are  $1\frac{1}{2}$  inches and 9 inches. The pulleys may be obtained from Central Die Casting and Manufacturing Co., Chicago, as 150A and 900A, respectively. Length of belt is  $33\frac{1}{2}$  inches. It is a V belt, Goodrich, FHP No. 1340.

## A METHOD FOR MEASURING THE TENSILE STRENGTH AND STRETCH OF PLASMA CLOTS\*

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BROOKLYN, N. Y.

IN THE course of experiments on autologous plasma clot suture of nerves,<sup>1</sup> a somewhat crude but simple method of testing the tensile strength and stretch of clots formed in vitro was devised. This technique has since been improved and in its present form has given fairly consistent measurements which have aided in the determination of the most suitable types of plasma clot for use as nerve suture material. Since the method may be of use in other fields of investigation, particularly the hematologic, a description of it is considered advisable. Careful search of the literature has disclosed previous methods for measuring the tensile strength and stretch of plasma clots.<sup>2, 3</sup> However, the present method, which was independently evolved, appears simpler, more readily constructed, and seems to us more suitable for accurate determination of these values.

Blood is drawn in a chilled syringe coated with mineral oil and is immediately transferred to paraffin-lined test tubes packed in ice. The tubes are placed in 250 c.c. metal cups filled with ice and are centrifuged for from five to seven minutes at 2,750 r.p.m. Centrifugation for longer periods often results in clotting of the plasma. On removal from the centrifuge the tubes are at once repacked in ice, and the supernatant unmodified plasma is ready for use. If blood to which an anticoagulant has been added is used, it is chilled and centrifuged in an identical fashion.

One cubic centimeter of the plasma is pipetted into a test tube of 8 mm. inside diameter in a water bath at 37° C. and allowed to clot. When a clotting agent is used, it is placed in the test tube in the water bath for a brief interval before addition of the plasma so that the temperatures may be as constant as possible throughout. After coagulation the clot is withdrawn from the tube by touching the tip of a heated platinum wire to the upper surface. The clot adheres to the wire and can easily be lifted out. When clot retraction has not occurred and the clot adheres to the test tube, a fine cool platinum wire is run carefully around the clot to loosen it.

The upper end of the clot is grasped in the rubber-sleeved jaws of a small hemostatic clamp held horizontally by means of a support, clamp holder and clamp. A common pin is pushed through one of the rubber sleeves and points to the zero mark of a millimeter rule held vertically by another clamp affixed

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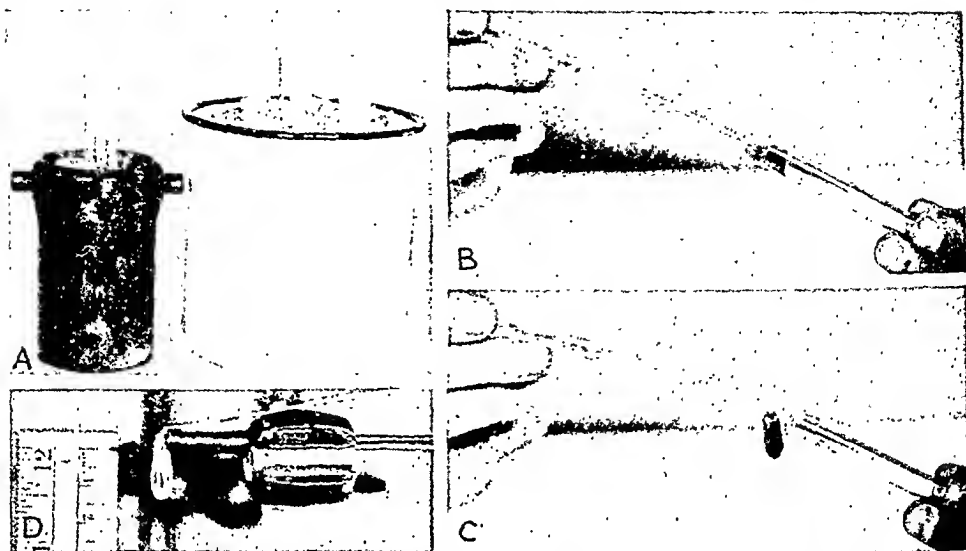


Fig. 1.—A, Metal centrifuge cup and enamelware pot containing test tubes packed in ice; B and C, removal of plasma clots from test tubes by heated platinum wire; D, grasping clot in clamp.

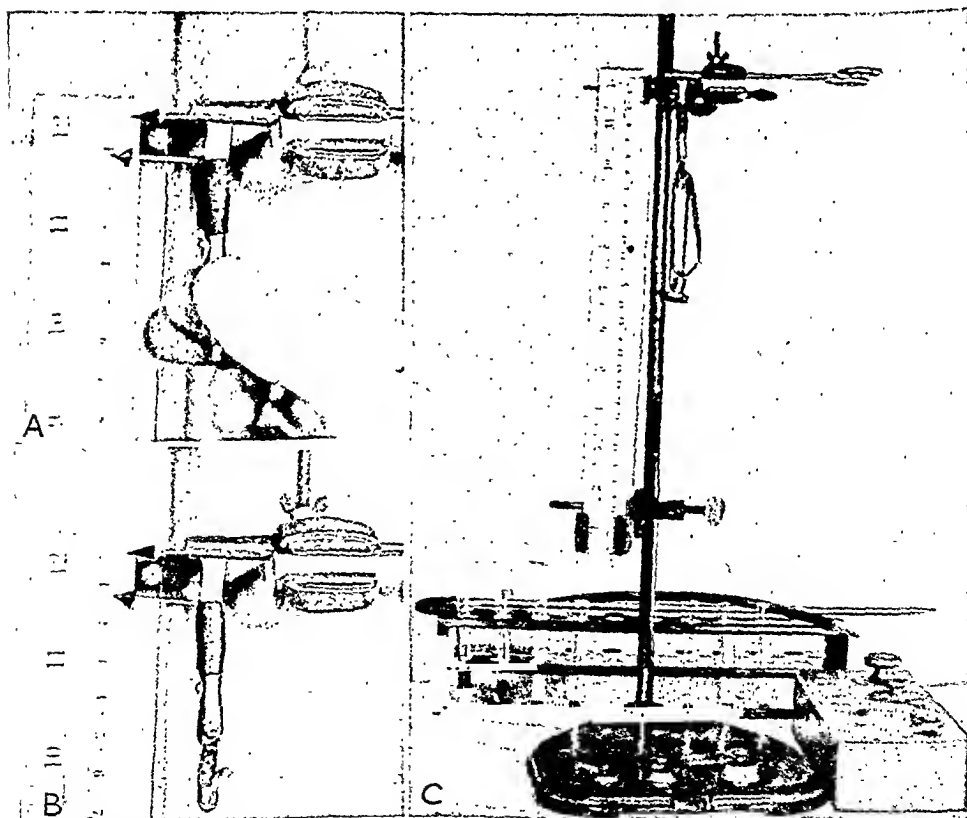


Fig. 2.—A, Application of clip to clot, leaving 1 cm. free for testing; B, stretch of 1.5 mm. following application of clip; C, use of apparatus for measuring tensile strength and stretch of clots. Clot shown supports clip and weights totaling 15 Gm. with stretch of 4.5 mm.

to the same support. The rule is just behind and to the side of the clot. The end of the pin pointer is fixed to the zero mark of the rule with a drop of collodion. This zero mark is permanently adjusted to correspond to the junction of the clot and clamp (Fig. 1). A light "radio clip" with the cylindrical wire guide cut open and bent to form a hook and with rubber-sleeved flattened jaws is clipped to the lower end of the clot. One centimeter of free clot is left for testing. Through one of the rubber sleeves of the clip is another common pin which indicates a mark on the rule level with the junction of clot and clip. A heavy silk thread is knotted about the upper pointer, strung parallel to the rule, and fixed to the clamp below. This serves as a guide for the lower pointer which runs between the rule and the thread and also serves to damp the swing of the clip which results from the addition of weights. The stretch resulting from the application of the clip (weight 4.0 Gm.) and each additional weight load is measured as the increase in distance between the two pointers. Rubber bands (weight 0.5 Gm.) are slit so as to slip over the heads of weights, and the weights are thus gently added to the hook of the clip at intervals of fifteen to forty-five seconds until the clot breaks (Fig. 2). It was found convenient to remove all the weights at 40 or 45 Gm. and to substitute for them, adding the next increment, a single 50 Gm. piece. At this point the tendency of the clot to approximate its original length is recorded. This may be repeated at 90 to 95 Gm. by adding a single 100 Gm. piece after removal of the weights. A cotton padded drinking cup may be placed beneath the weights to catch them and the clip when the clot breaks. The tensile strength of plasma clots from 8 healthy adult human males was found to average 44 Gm. The values obtained from ill persons were frequently considerably higher. The stretch seems to be directly proportional to the weight load; each 10 Gm. increment causes an average stretch of 2 to 3 mm. The final increment causes a greater rapid stretching which results in the break of the clot. Further detailed studies of tensile strength and stretch are being carried out.

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## METHOD OF SUSPENDING BOTTLE\*

E. E. MYERS, M.D., PHILIPPI, W. VA.

**A**N EASY method for suspending an inverted bottle as for use in a Wangenstein suction apparatus,<sup>1</sup> etc., is described.

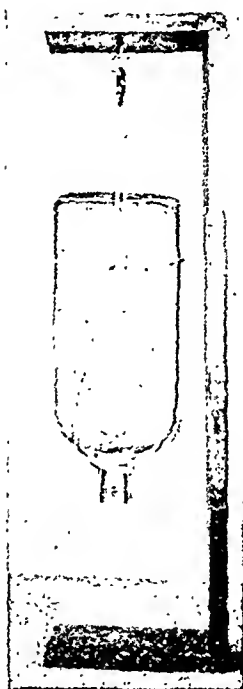


Fig. 1.

Either Venetian blind cord or suspender cord is satisfactory. A piece about 3 feet in length (for a gallon bottle) is used to make a ring around the bottle with three loosely tied knots evenly spaced. A second piece of cord, about 10 feet long, is started down through one of these loops, brought around the neck of the bottle, crossed over and brought back up through the next loop. It goes through the iron ring (harness ring  $1\frac{1}{4}$  inches in diameter) which is to suspend the bottle, passes down through the third loop and continues as above until there are two ropes through each loop. The ends of the rope are tied together and the knots in the rope around the bottle are drawn tightly.

Fig. 1 shows the appearance of the completed suspension.

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\*From the Myers Clinic, Philippi.  
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## PROTECTIVE CAPS FOR GLASS TISSUE GRINDERS\*

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TISSUE grinders consisting of paired tubes one within the other in which tissue fragments or bacterial clumps are emulsified are widely used in virus and bacteriologic work. The tubes are roughened and are made to fit snugly by grinding with abrasive powder.

Two types are principally used: (1) The TenBroeck grinder† made of heavy pyrex glass, and which is designed mainly for manual manipulation, and (2) those more or less similar in design to that described by Hanks, the plungers of which are activated by a mechanical rotary device.<sup>2-4</sup>

The use of grinders is accompanied by the risk of contamination of the material being ground by air-borne bacteria and of contamination of the operator with the material through splashing, etc.

The apparatus of Corper and Cohen<sup>3</sup> and that of Hanks<sup>4</sup> have devices to reduce the chances of contamination. The present report is concerned with a simple means of preventing contamination of the material and of protecting the operator when either the TenBroeck or Hanks type of grinder is used.

Devol gum rubber caps, such as are used for nursing bottles or centrifuge tubes, are selected which will fit over the mouth of the receptacle tube. A hole is punched in the center of the cap with a cork borer and the shank of the plunger is pushed through it. The hole should be of such size that the shank of the plunger fits snugly but without binding.

The grinder parts are wrapped separately for sterilization. The receptacle portion is plugged with cotton and may be dry sterilized. The plunger part, because of the rubber cap attached, must be autoclaved. After inserting the material to be emulsified, the lip of the receptacle tube is flamed, the plunger is inserted, and the cap is fitted over the outer tube with aseptic precautions.

The use of the protective cap is advantageous in grinding tissues by hand, since considerable manipulation for a time may be necessary and abundant opportunity for contamination is present.

Likewise, a protective device is desirable when cultures or tissues are emulsified using a mechanical rotor. The gum rubber caps described are more convenient than the holed cork stoppers used by Hanks, since diluting fluids can be aseptically introduced through the cap without stopping the motor by means of a syringe and hypodermic needle after rubbing the surface with alcohol.

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\*From the Laboratories of Bacteriology, University of Notre Dame, Notre Dame.  
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†Obtained from Scientific Glass Apparatus Co., Bloomfield, N. J.

## A SIMPLE TEST TUBE RACK AND COMPARATOR\*

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THE rack for test tubes is very simple and space saving, as can be seen from Fig. 1. If a test tube without a lip is desired, the lip can be easily substituted by a rubber band placed around the top of the tube. Should it be necessary to observe the lower part of the test tubes for extended periods of time, a detachable block may be placed to support each end of the rack (Fig. 2).

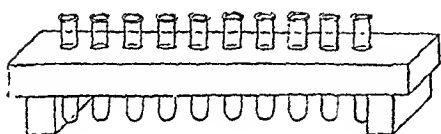


Fig. 1.

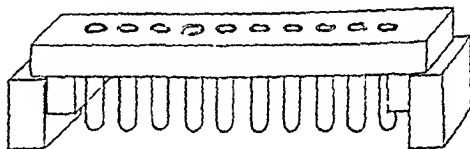


Fig. 2.

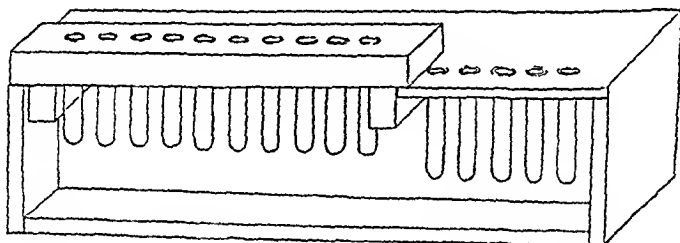


Fig. 3.

Further, the rack can be used to complement a light box for comparative readings and thus is a part of a simple comparator for color intensity or for measuring the brightness of light reflected by the particles in suspension in a tube. It was used in our studies with the toluidine blue reaction for heparin.<sup>1, 2</sup> The interior of the light box is painted white and contains two fluorescent bulbs of sufficient length to fill the longitudinal axis of the box. Their light is reflected through a plate of milk glass and thus illuminates the samples under test. The box can be used also as a titration light.

Fig. 3 illustrates the comparator with a permanent rack for tubes containing standard solutions and a support for the sample rack described above. Both rack and comparator are constructed from wood and are, therefore, inexpensive to manufacture.

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\*From the Hixon Laboratory for Medical Research, University of Kansas.  
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# AN APPARATUS FOR THE SIMULTANEOUS MEASUREMENT OF INTRAMUSCULAR AND VENOUS PRESSURE\*

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THE use of Henderson's modification of the instrument devised by Kerr and Scott<sup>1</sup> for the measurement of intramuscular pressure has certain drawbacks. (a) It requires the use of two men, both of whom must be trained in its use. (b) Especially difficult and a source of most errors is the reading of the end point when the meniscus begins to move in the capillary tube. (c) The need to refill constantly the capillary tube with sterile saline when frequent readings are desired.

Principally, the shortcoming of this method is the need for manpower greater than our team could furnish when simultaneous venous pressures were desired.

Tests conducted with the Henderson apparatus and our modification of the instrument by Wells, Youmans, and Miller<sup>2</sup> convinced us that the macroscopic gravity method for the measurement of the intramuscular pressure gave comparable results.

For convenience, the venous manometer and the intramuscular pressure manometer are mounted together. The sections can easily be disengaged for sterilizing (Fig. 1). The reservoir (*Rr*), which holds 50 c.c. saline, is connected through a glass stopcock (*S*) and a T tube to a 5 mm. glass tube with a 3 mm. bore (*M*). The outlet is controlled by a screw clamp (*Sc*) over a rubber tube which holds the female portion of a metal-connecting link (*C*). The male portion is inserted into rubber tubing, which in turn is connected to a needle adapter, and the 26 gauge needle which is inserted into the muscle.

TABLE I  
INTRAMUSCULAR AND VENOUS PRESSURE READINGS OVER A PERIOD OF 60 MINUTES

| PATIENT | TIME (MIN.) | INTRAMUSCULAR<br>PRESSURE (MM. H <sub>2</sub> O) | VENOUS<br>PRESSURE (CM. H <sub>2</sub> O) |
|---------|-------------|--|---|
| P       | 0           | 82   | 6.9                                       |
|         | 7           | 84   | 6.6                                       |
|         | 12          | 80   | 6.7                                       |
|         | 17          | 80   |   |
|         | 20          | 74   |   |
|         | 30          | 77   | 7.9                                       |
|         | 35          | 73   | 8.1                                       |
|         | 45          | 73   | 7.9                                       |
|         | 60          | 73   | 8.2                                       |

\*From the Cedars of Lebanon Hospital, Los Angeles.

N. J. Aided by a grant from the Ciba Pharmaceutical Products, Inc., Lafayette Park, Summit,

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The venous manometer is similarly constructed, but the tubing of the funnel and the manometer is split to facilitate sterilizing. The manometer and the reservoir tubes are connected by standard glass or metal-connecting links (Fig. 2).

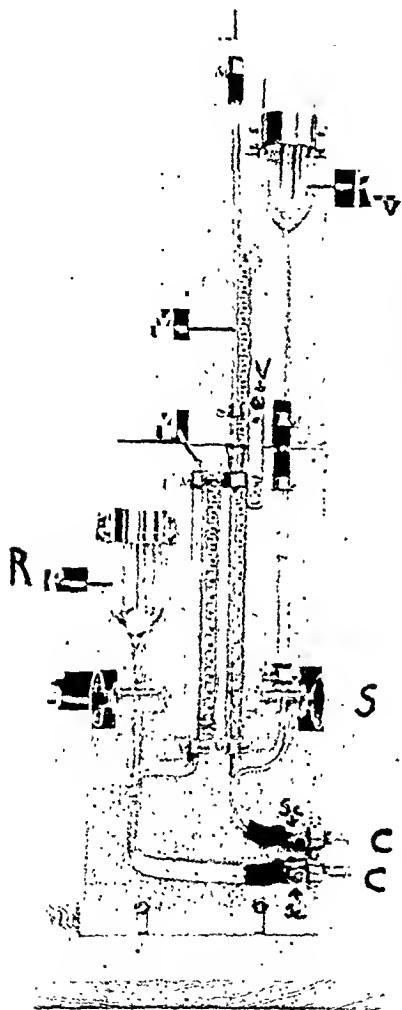


Fig. 1.

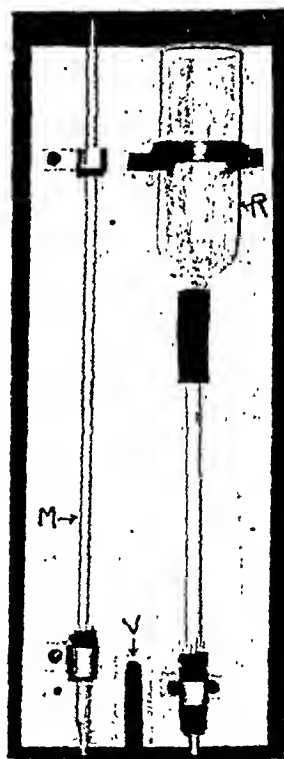


Fig. 2.

Fig. 1.—Apparatus for the simultaneous measurement of intramuscular and venous pressure, assembled after sterilizing.

Fig. 2.—Upper part of apparatus removed to show glass and metal connections and slot "V."

A vertical piece of metal (*V*) is mounted front and back to reinforce and give rigidity to the extension piece which is slotted (Fig. 2) to slip over the screw that is tightened lightly when the apparatus is assembled.

The venous reservoir (*Rv*) is filled with 3 per cent sodium citrate and the venous pressure needle is gauge 18.

The intramuscular reservoir (*Rir*) is filled with saline. The special slotted needle described by Henderson<sup>1</sup> is inserted into the muscle. The zero level of

the apparatus above the floor is determined by means of a water air bubble level, attached to a stiff steel tape (Fig. 3).

The level of the needle in the vein and in the muscle and the level of the auricle are obtained by the same means. The manometer tubes are filled from the reservoir, and are permitted to attain their level by gravity. It takes about five minutes for the fluid to equilibrate with the muscle and two to three minutes in the vein. Thus continuous readings of the intramuscular and venous pressures can be made at five-minute intervals.

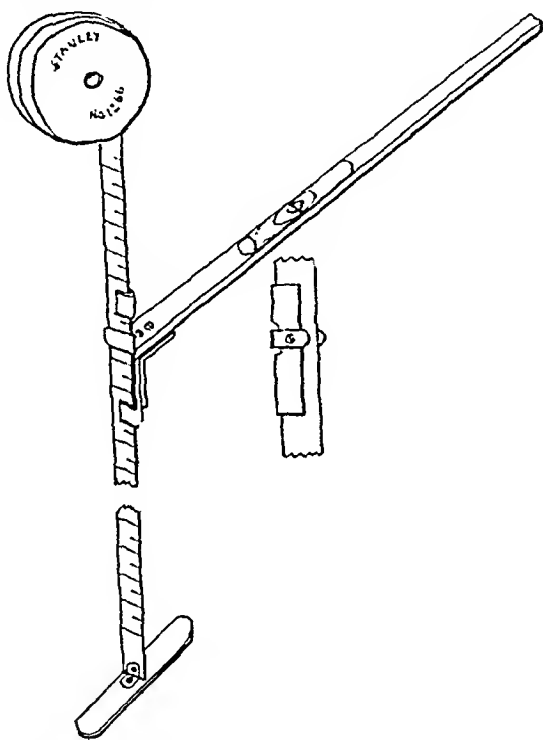


Fig. 3.—Steel tape and water level arrangement for obtaining zero point of apparatus, auricular level, and levels of the intramuscular and intravenous needles.

Difficulty with the reading may be had with the first intramuscular pressure reading. It may be necessary to pull the needle back or to insert it deeper to obtain a free fall of the fluid level in the manometer. A second and a third check reading will ascertain the workability of the apparatus. Thereafter, readings may be obtained over a long period of time merely by refilling the manometer tube from the reservoir to a level above the value of the intramuscular pressure and allowing it to fall by gravity. Likewise, the vein is flushed with 3 per cent sodium citrate from time to time to keep the needle clear, and continuous readings may be taken at will.

It will be found that intramuscular pressure readings will vary over a range of 10 mm., and venous pressure readings over a range of 2 cm. in any series of readings (Table I). The latter will vary only 1 cm. if the patient is quiet, not straining or coughing, but will vary over 2 cm. range if the patient is apprehensive.

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## AN IMPROVED ROUTINE TISSUE STAINING TECHNIQUE FOR FORMALIN-FIXED TISSUES<sup>2</sup>

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HENRY S. BLANK, A.B., M.D., COLUMBIA, S. C.

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AS A MATTER of convenience, time, and economy the time-honored routine tissue stain has been that of hematoxylin and eosin, almost to the exclusion of any other. The technique is simple, even for inexperienced hands; the results are adequate for routine diagnosis of surgical and necropsy pathology. The stain has its limitations, however, particularly in the presentation of certain histologic and pathologic structures. The chromatic expression of these structures in stained sections has given rise to a host of special stains which are individually applicable. Lack of time, material, and technical assistance has limited the use of these techniques. This is especially true of smaller pathologic laboratories. It follows that any technique that will give better and more extensive detail with a minimum of complexity will be welcomed.

Of the several other generally advocated routine tissue staining techniques, the Mallory eosin or phloxine methylene blue procedure has few peers. However, the effective presentation of histologic detail by this method is beset by several difficulties. Among these are the more or less axiomatic requirement of Zenker fixation, length of staining time, individual differentiation of slides under the microscope, and finally, lack of uniformity of results except in expert hands.

It was the aim of this laboratory to effect a staining technique that had the advantages of the methylene blue eosin system with none of its disadvantages.

### GENERAL PRINCIPLES

Ohlmacher's discovery of the mordanting action of formaldehyde on coal tar dyes was utilized in the preparation of a methylene-blue-azure mixture.<sup>1</sup> Giemsa stain was added to the methylene blue as a source of the azures. It appears to contribute a generally wider chromatic scale by giving additional variation in the shades of blue.

Mercurochrome 220, commonly used as a disinfectant, replaced eosin. This dye is dibrom-oxymerecury-fluorescein and is closely related to eosin which is tetra-bromfluorescein. Conn<sup>2</sup> quotes Baldwin as stating that it may be used in

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\*From the Pathological Laboratory of the Veterans' Administration Facility, Columbia.  
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It was determined that the 2 per cent aqueous solution gave far too brilliant results, and that a dilution of 0.1 per cent was more than adequate. The stain has an apparent specific affinity for cytoplasmic structures and is not easily washed out. The diffuseness of staining, so characteristic of cosin, is quite absent.

## REQUIRED STAINS

1. Hematoxylin, Delafield.
2. Methylene blue—0.5 Gm. dissolved in 10 c.c. of absolute alcohol diluted to 100 c.c. with 4 per cent neutral formalin.  
Giemsa stain—stock solution—4.5 c.c. diluted to 100 c.c. with 4 per cent neutral formalin.  
Mix both stains. This mixture forms a stock solution to which is added 1 Gm. of borax. Dilute 1:20 for use.
3. Mercurochrome 220—2 per cent stock solution 5 c.c.  
Alcohol 95 per cent 25 c.c.  
Water To make 100 c.c.

### STAINING PROCEDURE

1. Deparaffinize formalin-fixed tissue sections in two changes of xylol.
2. Absolute alcohol —one minute.
3. Alcohol, 95 per cent—one minute.
4. Alcohol, 70 per cent—one minute.
5. Water —one minute.
6. Hematoxylin, Delafield—five minutes.
7. Wash in two changes of water to remove excess stain.
8. Decolorize until rose colored in water to which several drops of concentrated hydrochloric acid have been added.
9. Wash in water.
10. Wash until blue in water to which several drops of strong ammonium hydroxide have been added.
11. Wash in water.
12. Mercurochrome solution—one minute.
13. Wash in water to remove excess stain.
14. Methylene blue—azure stain—two minutes.
15. Wash in water to remove excess stain.
16. Decolorize in 95 per cent alcohol until no more color is seen to come off in drippings, about fifteen to thirty seconds.
17. Wash briefly in 95 per cent alcohol and complete dehydration in two changes of absolute alcohol.
18. Clear in two changes of xylol.
19. Mount in balsam.

It is desirable to filter the mereurochrome before using, to remove the bright golden seum which forms on standing for any length of time. A 0.1 per cent aqueous solution has been found satisfactory, without the addition of

alcohol, but the latter appears to give slightly better results. No particular improvement was noted by the addition of colophony to the differentiating alcohol.

Staining by this technique appears to give greater nuclear detail than is observed by hematoxylin and eosin. Fibrous tissue is sharply differentiated from muscle tissue. Inflammatory and blood elements are exceptionally prominent and present detail in both nucleus and cytoplasm. Other structures stain as in the Mallory technique.

#### SUMMARY

A new staining technique is presented which is suitable to routine tissue sections fixed in formalin. It is simple, does not require individual microscopic differentiation of sections, and produces excellent and uniform results even in inexperienced hands.

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## A NEW DEVICE FOR THE APPLICATION OF SCALP ELECTRODES IN ELECTROENCEPHALOGRAPHY

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THE device consists of a spring air valve seated in a brass or plastic casing. Fig. 1 shows in longitudinal section the essential design characteristics and mechanism of the device. The total weight of the electrode applier, with brass casing, is less than 15 Gm.

#### METHOD OF USE

The scalp surface is prepared according to standard procedures. The solder pellet electrode is then held in position by placing the valve pin on the electrode and making gentle pressure on the thumb rest of the electrode applier. A drop or two of collodion is then allowed to flow over the electrode. To hasten the setting of the collodion, it is necessary to disperse its volatile solvents. This is readily accomplished by a blast of air from the device. A moderate increase in the pressure exerted on the thumb rest of the electrode applier opens the valve and allows the compressed air to escape through the applier outlet. Three to five pounds per square inch of pressure is quite adequate for efficient drying. Since only low pressures of air are required, the electrode applier can be con-

\*From the Laboratories of St. Elizabeths Hospital, Washington, D. C.

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needed to the compressed air-reducing valve (from tank or line) by means of thin-walled rubber tubing. This adds much to the general ease of manipulation of the device.

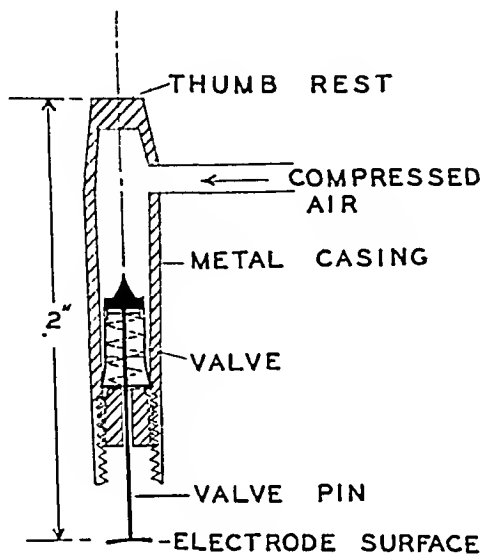


Fig. 1.

#### CONCLUSIONS

This electrode applicator simplifies the technique of electrode application for the following reasons:

1. Ease of manipulation resulting from its compactness and lightness.
2. Efficiency of operation.
3. More firm and more uniform application of electrodes result from the minimum pressure required to open the air valve.
4. Rapidity of electrode application because of the proximity of the air blast to the applied electrode.

## CHEMICAL

### SIMPLIFIED OXYGEN ANALYZER FOR OXYGEN TENTS\*

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THE importance of oxygen concentrations in the therapeutic uses of oxygen has received considerable emphasis. In spite of this, many oxygen tents are not controlled by oxygen concentration determinations.

Many oxygen tent analyses were made and the majority of the determinations showed oxygen concentrations to be from 30 to 45 per cent. After the nurses were instructed to flood the tents more frequently, the concentrations were somewhat higher but were still not satisfactory. It was also apparent that regulation of the concentration by a technician to 50 per cent or over three times a day would not maintain the concentrations satisfactorily between his visits because of the nursing care which necessitated opening the tents. It was concluded, therefore, that oxygen analysis by the nurses was necessary in order to maintain the desired concentrations.

The following criteria were considered essential for an oxygen analyzer suitable for use by nurses: 1. Use of equipment familiar to nurses. 2. No adjustment of fluid level. 3. Absence of valves. 4. Reasonably foolproof. 5. Inexpensive. 6. Accuracy within plus or minus 2 per cent.

The analyzer described below was devised. The fifteen which were built and used for two years with satisfactory results fulfilled the above-mentioned criteria.

#### DESCRIPTION

The analyzer consists of a 200 c.c. glass jar (see Fig. 1) filled with No. 30 pure copper cloth. A No. 11½ rubber stopper is drilled to hold a glass tube 8 mm. in external diameter and 10 inches long, and to hold firmly the tip of a standard 10 c.c. syringe. The glass tube extends from the bottom of the jar high enough so the fluid does not overflow. The lower end of the tube is constricted by heating and the upper end is closed with a small toy balloon.

The scale consists of a strip of paper cemented to a strip of light brass and held on the tube with rubber bands. The scale is calibrated by injecting with the syringe the following amounts of air, and after the fluid stops dropping, the level is marked at the concentration as indicated: 10 c.c. of air, 21 per cent; 8.75 c.c., 30 per cent; 7.5 c.c., 40 per cent; 6.25 c.c., 50 per cent; 5 c.c., 60 per cent; and 3.75 c.c., 70 per cent.

The solution in the copper-ammonia-chloride reagent, recommended by Badger,<sup>1</sup> consists of equal amounts of water and concentrated ammonium hy-

\*From the Oxygen Therapy and Clinical Research Departments of St. Luke's Hospital and the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

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dioxide (28 per cent) saturated with ammonium chloride. This solution does not give off enough ammonia to be objectionable. As it becomes exhausted, the solution forms a yellow precipitate which can be dissolved with a small quantity of the fresh solution.

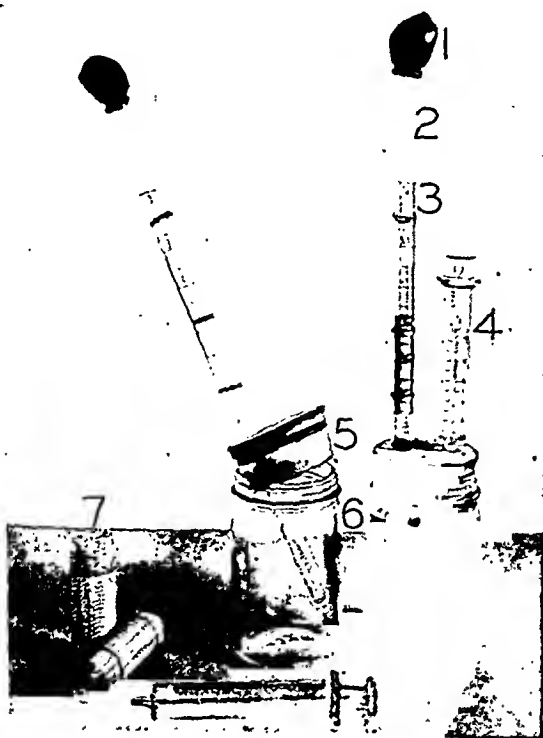


Fig. 1.—Simplified oxygen analyzer. 1, Toy balloon; 2, glass tube; 3, scale; 4, syringe; 5, rubber stopper; 6, glass jar; 7, roll of copper cloth.

#### TECHNIQUE

A 10 c.c. syringe, moistened occasionally with water, is attached to the oxygen sampling outlet or catheter extending into the tent and flushed four to six times. The plunger is withdrawn to the 10 c.c. mark, the syringe is detached, and the tip is covered with the finger. The tip is inserted into the hole in the rubber stopper, care being taken not to let the plunger move during the procedure. The plunger then is pushed down gently and twisted until it sticks. After the fluid level in the tube is stationary (within twenty seconds to one minute), the concentration is read on the scale. The syringe is left in place until the next analysis.

Once every day or two the scale is checked by an injection of 10 c.c. of air. If the level does not stop at the 21 per cent mark, the scale is adjusted as necessary. This, like the replacing of the solution, should be done by a technician or by the supervisor of the oxygen therapy.

#### DISCUSSION

An oxygen tent is no better than the oxygen concentration. Oxygen administration is similar to administration of any medicine, and the significance of

the dosage should be recognized and controlled. Analysis by a technician at intervals is valuable, but inasmuch as the tent may have to be opened for nursing attention between analyses, there are periods when the concentration drops.

This analyzer is designed for the nurse to use. She is instructed to make an analysis each time the tent has been opened and closed, and to adjust immediately the concentration to 50 per cent or over. In addition, the concentration is determined every hour. This frequent testing of the concentration also makes the nurse realize that frequent unnecessary opening of the tent is detrimental to the patient.

The time required for the analysis is almost negligible. Withdrawing the sample and injecting it into the analyzer requires less than thirty seconds, and the nurse can return in a minute or two and read the concentration.

The cost of the material for making the analyzer itself is small; the syringe, furthermore, is a standard hospital item. The apparatus can be made in any laboratory equipped with the necessary hole cutters and metal shears. This inexpensiveness makes it possible to have several on hand. When the solution becomes exhausted, another can be substituted.

#### SUMMARY

An oxygen analyzer is described, which has been designed specifically for use by nurses. The analyzer is reasonably accurate, simple to use, inexpensive, and can be made in the ordinary laboratory.

Analysis of the oxygen concentration after each time the tent is closed and at frequent intervals increases the efficiency of the therapy.

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# THE SPECTROPHOTOMETRIC DETERMINATION OF PHOSPHORUS AND CHOLESTEROL\*

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HAROLD H. WILLIAMS, PH.D., DETROIT, MICH.

RECENT advances in the development of spectrophotometric instruments for precise color measurement make it possible to attain equal or greater accuracy in chemical analysis and to employ procedures which have been hitherto unavailable. Furthermore, they offer the advantage of increasing the number of determinations that may be performed in a given time. This paper presents the adaptation of phosphorus and cholesterol methods to the spectrophotometer.

## PHOSPHORUS DETERMINATION

The boundaries of quantitative biochemistry have been greatly extended by Van Slyke and his associates through the development and application of the gasometric technique for the microdetermination of organic carbon.<sup>1-3</sup> The principle of their procedure has been adapted successfully to microquantities of inorganic phosphate,<sup>4</sup> sulfate,<sup>5</sup> calcium,<sup>6</sup> and magnesium<sup>7</sup> by manometric combustion of their organic precipitates. The introduction and use of special combustion centrifuge tubes<sup>5, 6, 8</sup> permitting, without transfer, precipitation, washing, and combustion of the organic compound has simplified the method and expedited the time needed for carrying out the determination.

From the viewpoint of microtechnique and accuracy, the gasometric determination of phosphorus is ideal. The laboratory possessing a spectrophotometer, however, may use the present adaptation to advantage, since it facilitates with a similar degree of accuracy the analysis of large numbers of samples with less highly skilled and trained personnel, and eliminates any interference from contaminants such as atmospheric dust and carbon dioxide. Applying the following procedure, it has been possible to perform a large series of phosphorus analyses in one day whereas formerly two or three days were required.

The procedure of Kirk<sup>4</sup> is followed up to the washing of the precipitated phosphoric acid in the form of strychnine phosphomolybdate. An aliquot (usually 6 ml.) of the phosphoric acid sample is placed in an ordinary 15 ml. conical pyrex centrifuge test tube. Two milliliters of the strychnine molybdate are added dropwise with continuous shaking and allowed to stand not less than ten minutes.† The tube is centrifuged, the supernatant liquid is decanted, and

\*From the Research Laboratory of the Children's Fund of Michigan, Detroit.  
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†In the original procedure of Kirk the reagent is added rapidly to the sample and the contents of the tube are thoroughly mixed. The tube is then permitted to stand for twenty to forty-five minutes to complete the precipitation. If carried out by the above modification, there is saving in time since precipitation is complete at the end of ten minutes.

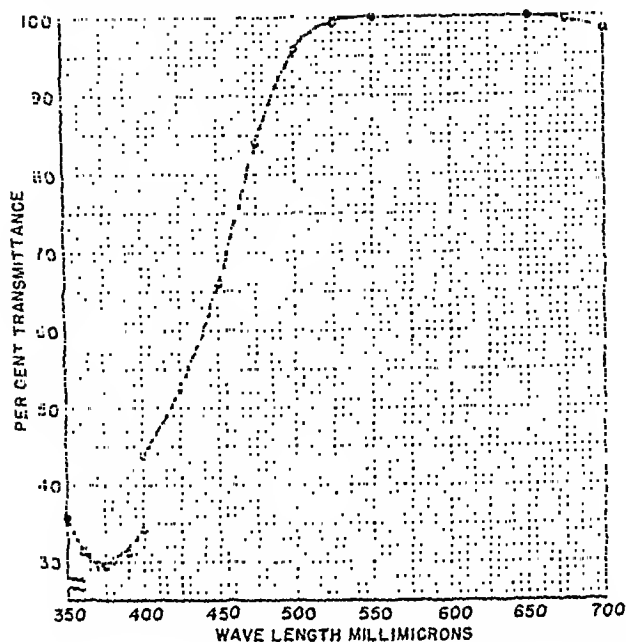


Fig. 1.—Transmittance wave length curve for color of strychnine phosphomolybdate in dilute sodium hydroxide. Filter No. PC 6 used from 350 to 400 mμ. Filter No. PC 4 used from 400 to 700 mμ. Concentration of phosphorus, 0.025 mg. in 100 ml.

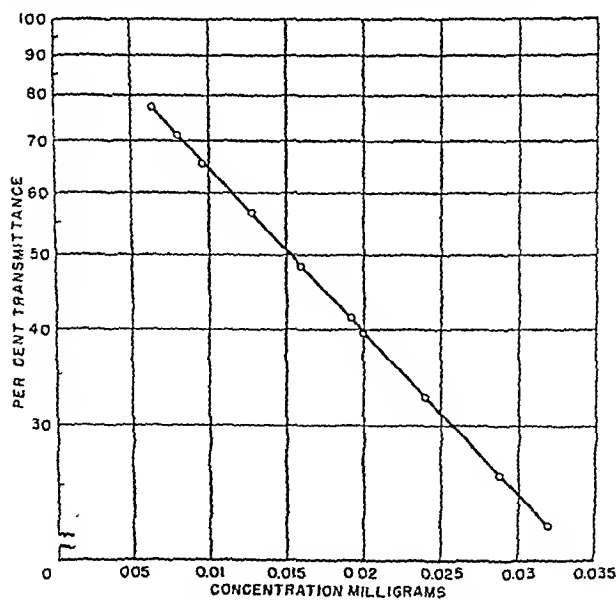


Fig. 2.—Concentration of phosphorus vs. transmittance.

the mouth of the tube is wiped free of any remaining liquid. Two milliliters of 1 per cent nitric acid are added, the precipitate is stirred with a glass rod, and the tube is centrifuged for five minutes. The washing with nitric acid is repeated twice (three washings in all); no stirring is necessary in the last wash. The precipitate is then dissolved in 2 ml. of 1 per cent sodium hydroxide, and

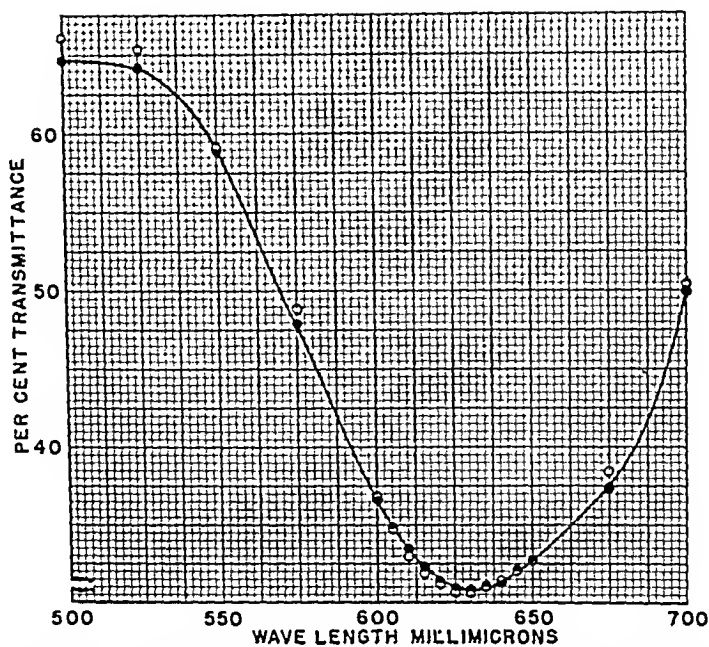


Fig. 3.—Transmittance wave length curve for color developed by cholesterol (clear circles) and cholesterol digitonide (filled in circles). Cholesterol concentration 0.6 mg. in 6 ml.

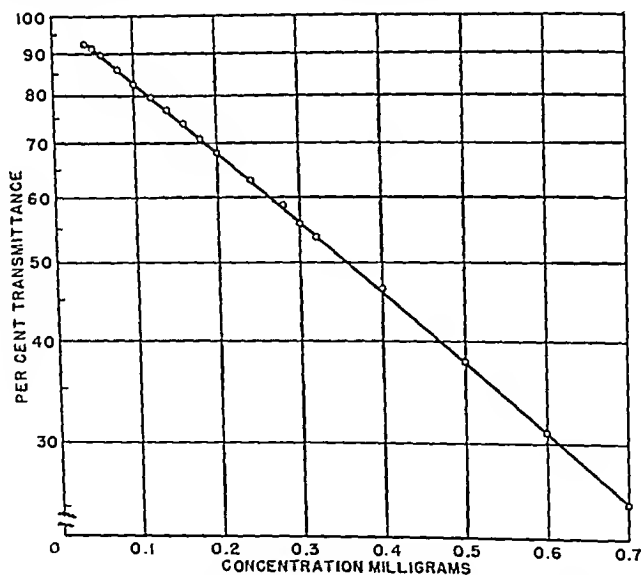


Fig. 4.—Concentration of cholesterol vs. transmittance. Maximum average deviation  $\pm 0.6$ .

the contents of the tube are completely transferred into a 100 ml. volumetric flask with distilled water. After making to volume, the sample is ready for color measurement.\*

Fig. 1 shows the spectral transmittance curve of the stryehnine phosphomolybdate in a dilute sodium hydroxide solution. From 400 to 550  $m\mu$  the

\*In the present study the Coleman Universal Spectrophotometer was used. Any similar device that will allow for the selection of light of the proper wave length should be suitable.

absorption decreases to zero and remains there until 650  $m\mu$  is reached. Above 650  $m\mu$  there is slight absorption. Since the maximum absorption between 400 to 700  $m\mu$  was at 400, the spectral properties of the solution were examined between 350 and 400  $m\mu$ . The maximum absorption was obtained at 375  $m\mu$ .

TABLE I

COMPARISON OF SPECTROPHOTOMETRIC AND GASOMETRIC DETERMINATION OF PHOSPHORUS

| PHOSPHORUS<br>MG. | GASOMETRIC<br>PHOSPHORUS<br>MG. | RECOVERY GASOMETRIC<br>PHOSPHORUS<br>PER CENT | TRANSMITTANCE*<br>PER CENT |
|-------------------|---------------------------------|---|----------------------------|
| 0.0064            | 0.00648                         | 101.3   | 77.0                       |
| 0.0080            | 0.00808                         | 101.0   | 71.0                       |
| 0.0096            | 0.0098                          | 102.2   | 65.3                       |
| 0.0128            | 0.01276                         | 99.6  | 56.4                       |
| 0.0160            | 0.01588                         | 99.3  | 48.3                       |
| 0.0192            | 0.01922                         | 100.1   | 41.4                       |
| 0.020             | 0.0197                          | 98.5  | 39.5                       |
| 0.024             | 0.0244                          | 101.6   | 32.6                       |
| 0.0288            | -                               | -   | 25.8                       |
| 0.032             | -                               | -   | 22.1                       |

\*Maximum average deviation  $\pm 0.9$ .

Table I shows the comparative values of a series of standards ranging from 0.0064 mg. to 0.032 mg. of phosphorus, as determined by both the spectrophotometric method and by the gasometric microtechnique. By plotting the transmission values against the quantities of phosphorus on semilog paper, a straight line results, as illustrated in Fig. 2, thus demonstrating that Beer's law is obeyed over the range of concentrations studied.

#### CHOLESTEROL DETERMINATION

There have been many procedures proposed for the determination of cholesterol. The microprocedure for the determination of free and combined cholesterol of Schoenheimer and Sperry<sup>9</sup> achieves a degree of accuracy which has previously been obtainable only with the classical macrogravimetric technique of Windaus. Because of the weak color intensity in the small volume required by the method, the use of this excellent and precise procedure, unfortunately, has been limited to only those investigators possessing a photometer. Most of the available photoelectric measuring devices are not equipped with cells of the proper depth to volume ratio to make accurate measurement of the limited amount of faintly colored solution.<sup>10</sup> The colorimeter has been employed for measuring the color, but it has not been altogether practical.<sup>9, 10</sup> The Coleman Universal Spectrophotometer, however, which employs a square cuvette giving a depth of approximately 1.3 cm. for a volume of 6 ml., has proved satisfactory, provided the cholesterol digitonide is dissolved in double the quantities of reagents, as recommended<sup>9</sup> for large precipitates.

The method up to the dissolving of the cholesterol digitonide for color measurement is exactly as given by Schoenheimer and Sperry,<sup>9</sup> including the more recent improvements recommended by Sperry.<sup>8, 10</sup> The cholesterol digitonide is dissolved in 2 ml. of glacial acetic acid, 4 ml. of acetic anhydride, and 0.2 ml. of concentrated sulfuric acid. After developing the color in accordance with the original method,<sup>9</sup> it is measured in the spectrophotometer employing the

630  $m\mu$  wave band. From the spectral transmittance curves of cholesterol and cholesterol digitonide, as shown in Fig. 3, it may be noted that the maximum absorption occurs between 620 and 635  $m\mu$  for both compounds. Noninterference of the digitonide with the light absorption of cholesterol is a decided advantage, as was pointed out originally.<sup>9</sup>

Transmittance values were determined for 0.04 mg. to 0.7 mg. of cholesterol, encompassing a range higher than that given in the original method (0.02 to 0.015 mg.). This wider range has been made possible by doubling the amounts of digitonin used for precipitation of the cholesterol. The 1 ml. of digitonin solution recommended<sup>9</sup> will precipitate the cholesterol completely up to and including 0.3 mg. Above 0.3 to 0.7 mg., however, 2 ml. of the digitonin solution are required to precipitate the cholesterol completely. If there is doubt as to the quantity of cholesterol in an unknown, 2 ml. of the digitonin solution may be used with as low as 0.1 mg. of cholesterol. A series of samples containing from 0.1 mg. to 0.3 mg. gave the same per cent transmittance values when precipitated with 1 ml. of digitonin solution as with 2 ml. Plotting the transmittance values against the concentration of cholesterol (Fig. 4), it may be noted that Beer's law operates over the range of concentrations studied.

#### SUMMARY

The gasometric phosphorus and the colorimetric cholesterol digitonide methods have been adapted successfully to use with the spectrophotometer without sacrificing original features of microtechnique, specificity, and accuracy. Phosphorus may be determined within the range of 0.006 to 0.3 mg., quantities similar to the gasometric method. For cholesterol it has been possible to enlarge the quantities (0.04 to 0.7 mg.) determined without loss of accuracy. As a result of these adaptations the above micromethods should find a wider application.

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## A MECHANICAL MANIPULATOR FOR SMALL PIPETTES\*

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IN THE course of some work on the chemical properties of native mucous secretion from the digestive tract, we encountered difficulty in delivering exact volumes of this extremely viscous material for microchemical analysis. Our analytical methods aim at a precision of better than 1 per cent, and they call for the delivery of 0.1 ml. samples with a considerable degree of reproducibility.

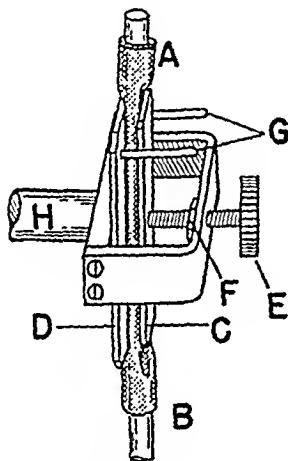


Fig. 1.—Manipulator for small pipettes.

Because of its extreme viscosity and tenacity, we found it necessary to draw the material up into the sampling pipette without overshooting the graduation as well as to deliver the sample with rinsing. Consequently, the usual oral method of manipulating a pipette was unsuited for this purpose, and it became necessary to employ a mechanical device. The following arrangement, embodying a simple mechanical principle, has proved so very efficient in our work, as well as for other purposes, that we describe it for the benefit of others, especially workers in clinical laboratories who frequently work with 0.1 ml. quantities.

Contrary to the usual procedure, the pipette and manipulator are supported rigidly in a ring stand. The vessel containing the material for analysis is supported underneath by one hand while the sample is being taken. Suction and delivery are both effected by mechanically controlled changes in the volume of air trapped in a small piece of rubber tubing. The upper end of this tubing (A) is closed with a piece of glass rod or with rubber cement; the pipette stem is at-

\*From the Laboratories of the Mount Sinai Hospital, New York City.  
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tached to its lower end at (*B*). The tubing is held between two rigid metal plates, the front one of which (*C*) can be driven gradually against the back one (*D*) by means of a screw with knurled knob (*E*). These plates are both 5 cm. long; their widths are 13 and 35 mm., respectively. The distance between the front of the supporting frame and the back plate is about 15 mm. A nut (*F*) soldered to the inside of the supporting frame serves to steady the movement of the screw without increasing the weight of the device. The pins (*G*) serve only to keep the front plate (*C*) from swerving from side to side, and the 15 cm. rod (*H*) supports the device on a ring stand.

Before the pipette is immersed in the liquid, the tubing must be compressed to a moderate degree; thereafter, gradual release of this pressure will serve to draw the liquid up to the graduation mark without overshooting the mark, and recompression will serve to deliver the sample as required. In order to insure complete filling and emptying of the pipette, the volume of air contained in the rubber tubing must be slightly greater than the capacity of the pipette. For 0.1 ml. samples we use a piece of red rubber tubing (inside diameter 4.5 mm., wall thickness 1 mm.) about 6 cm. long, exclusion of the upper and lower ends which are filled with glass. A similar device of slightly larger dimensions has been employed for pipettes of greater capacity.

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## SIMPLIFIED MICROMETHOD FOR BLOOD SULFONAMIDE DETERMINATION\*

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GILBERT M. JORGENSEN, M.D., ANCON, CANAL ZONE

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IN THIS report is described a modification of the diazotization method<sup>1</sup> for quantitative determination of unconjugated sulfonamides in blood. The modification permits the use of smaller quantities of blood and lessens the time required for performance of the test. In accomplishing these advantages only a small degree of accuracy has been sacrificed.

A reduction in the amount of blood required to only 0.02 c.c. has made possible blood sulfonamide determinations on small laboratory animals without sacrificing their lives. The method has been applied without difficulty for blood sulfonamide determinations on mice. It has been used for six months for routine determinations on children receiving sulfonamides, and has proved satisfactory.

A saving in time required for performance of the test has been accomplished by omitting the precipitation of blood proteins which permits the determination to be carried out in one vessel.

An Evelyn photoelectric colorimeter with filter 540 M has been employed, though other photoelectric colorimeters should serve equally well. For measuring the blood ordinary 0.02 c.c. "hemoglobin" pipettes have been used.

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The reagents required are glacial acetic acid, 0.1 per cent aqueous solution of sodium nitrite, 0.5 per cent aqueous solution of ammonium sulfamate, and a 1 per cent aqueous solution of N-(naphthyl) ethylenediamine dihydrochloride.

#### PROCEDURE

To 2 c.c. of distilled water in a colorimeter tube add 0.02 c.c. of capillary blood with a "hemoglobin" pipette. Add 2 c.c. of glacial acetic acid and 1 c.c. of a 0.1 per cent aqueous solution of sodium nitrite. Shake. After one minute or more add 1 c.c. of a 0.5 per cent aqueous solution of ammonium sulfamate. Shake. As soon as the bubbles have risen to the surface, insert the tube into the Evelyn photoelectric colorimeter after adjusting the tube holder for 6 c.c. volumes and inserting filter 540 M. To obtain the first of the two readings necessary, adjust the galvanometer string to 5.0 on the lower scale (the midpoint, sometimes designated 50), remove the tube, and read the galvanometer. This first reading (air setting) serves as a blank and also enables one to calculate the hemoglobin concentration.

After the first reading has been obtained, add 0.02 c.c. of a 1 per cent aqueous solution of N-(naphthyl) ethylenediamine dihydrochloride from a dropper calibrated to deliver that amount per drop. Shake. After three minutes or more adjust the galvanometer string to the first reading, insert the tube, and obtain the second reading.

Calculation of the concentration of the sulfonamide present involves the use of the following formula:

Concentration of sulfonamide =  $K (\log 5.0 - \log \text{second reading}) + C$ .  
K is a constant determined by using a hemoglobin-free solution of a sulfonamide of known concentration. C is a correction made necessary because of the presence of hemoglobin; within the range of hemoglobin values encountered, the correction necessary is approximately proportional to the hemoglobin concentration.

The concentration of hemoglobin may be calculated from the following formula:

Concentration of hemoglobin =  $K' (\log \text{first reading} - \log \text{first reading of hemoglobin-free blank})$ .

The correction  $C = K (\log 5.0 - \log \text{second reading}) - K'' (\log \text{first reading} - \log \text{first reading of hemoglobin-free blank})$  may be incorporated into the first formula as follows:

Concentration of sulfonamide =  $K (\log 5.0 - \log \text{second reading}) + K (\log 5.0 - \log \text{second reading}) - K'' (\log \text{first reading} - \log \text{first reading of hemoglobin-free blank})$ . K, K', and K'' may be determined from standards of known concentration. The application of the above formula appears rather laborious; however, with the aid of a calculating machine one may prepare in a few hours a table listing the values for all galvanometer readings.

The correction, C, is not large; for sulfanilamide it amounts to about 1 per cent per gram of hemoglobin. For routine determinations, if an error up to 10 per cent is acceptable, one may simplify the standardization and the prepara-

tion of a reference table by omitting C from the formula and determining a constant  $K'''$ , using blood of average hemoglobin concentration in addition to a standard sulfonamide solution. A blank is not necessary except for the determination of the constants.

The most likely source of error results from the occasional adherence of bubbles to the side of the colorimeter tube. If the tubes are cleaned with cleaning solution, bubbles seldom adhere to the glass, and when present, they usually may be dislodged by shaking the tube.

#### REFERENCE

1. Bratton, A. C., and Marshall, E. K., Jr.: New Coupling Component for Sulfanilamide Determination, *J. Biol. Chem.* 128: 537-550, 1939.

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## ITEM

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### Meeting of Scientific Photographers

The Biological Photographic Association will hold its Twelfth Annual Convention in New York City September 10, 11, and 12. Present-day methods of obtaining photographs for teaching and scientific records will be discussed in detail. Particular emphasis will be placed on the types of photographs needed in the present emergency. The Convention Chairman will be Mr. Joseph Haulenbeek, Illustration Division, Rockefeller Institute for Medical Research, New York City. Further particulars about the meeting and the program may be had by writing him.

The Biological Photographic Association is a national, nonprofit organization, formed to act as a clearing house for information on the methods of scientific illustration. Its members are scientific photographers, scientists interested in the application of photography to their fields, and the makers of precision photographic equipment. Information on various techniques, collected from experienced workers all over the country, is dispensed by means of the Association's Quarterly Journal, Travelling Loan Albums and Print Salons, and direct correspondence through the office of the Secretary. The 1942 Salon of medical and biological prints will be on view at the Convention.

# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

HEMOGLOBINOMETRY, Rimington, C. Brit. M. J. 1: 177, 1942.

The following method is described:

A 1:500 dilution of blood in decinormal sodium hydroxide is made, using a standard or calibrated pipette. This dilution may, of course, be made in two steps, if it is preferred, as in the worked example quoted below. After a few minutes at room temperature a convenient volume, such as 10 c.c., is measured into a small flask, and one-fifth of the volume of pyridine is added. Upon mixing by gentle rotation the conversion of the hemepigments into the alkaline parahematin is completed within a few seconds; hence there is no necessity to allow the original blood dilution to stand for ten, twenty, or even forty minutes before proceeding, or alternatively to heat in a boiling water bath, as is required when the alkaline hematin method is used.

A pinch of good quality sodium hydrosulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) is now added to the mixture, and the contents of the flask are swirled gently until the solid has dissolved. The immediate color change to pink denotes the conversion of the pigments into pyridine hemochromogen. Without undue delay (to guard against reoxidation) the solution is transferred to a photoelectric colorimeter cell, and the light absorption in the region 550 to 555  $\mu$  is measured, using a suitable filter (e.g., Ilford spectrum filter No. 605—yellow green). From the calibration factor, obtained by using known weights of pure hemin instead of blood and proceeding as above, the hemoglobin content of the blood is then calculated.

Molecular weight of hemin,  $\text{C}_{34}\text{H}_{32}\text{O}_4\text{N}_4\text{FeCl}$  = 651.4

Iron content of hemoglobin = 0.334 per cent

Therefore, Molecular weight of hemoglobin = 66,890

$$\frac{\text{Hemoglobin}}{\text{Hemin}} = \frac{66,890}{4 \times 651.4} = 25.66$$

Should it be contended that the error involved in calculating the molecular weight of hemoglobin from its iron content is undesirably large, the results might alternatively be expressed in terms of hemin or hematin. The extension of pyridine-hemochromogen prepared from crystalline hemin and using a photoelectric absorptiometer with Ilford No. 605 filter and 1 cm. cell was found to be 0.108 at a concentration of 1 mg. per 100 c.c.

Should no photoelectric colorimeter be available, the concentration of the pyridine-hemochromogen solution may be determined by one of the alternative methods, such as color comparison in a colorimeter with a standard prepared from hemin (standard solutions of hemin in alkali should be kept in the dark) or dilution while viewing with a hand spectroscope until the intensity of the absorption band matches that of a standard, as described by Roets (1940). This last method is certainly the most simple and rapid, although not quite so accurate as the use of a colorimeter. Pure hemin is easily prepared according to Gattermann (1937), but should be recrystallized as follows:

Five grams of hemin are dissolved in 15 c.c. of pyridine, and 40 c.c. of chloroform are added. The solution is passed through a No. 3 or No. 4 sintered-glass filter to remove any protein and is run into a mixture of 250 c.c. of acetic acid plus 5 c.c. of saturated sodium chloride plus 4 c.c. of concentrated hydrochloric acid, which is maintained at water-bath temperature. After cooling to room temperature the crystals are filtered and washed successively with 50 per cent acetic acid, water, alcohol, and ether, and then dried.

The author has used the pyridine-hemochromogen method with equal success for specimens of plasma and urine containing hemoglobin, myohemoglobin, or mixtures of these with their derivatives. It is only necessary to adjust the initial dilution so that the concentration of pigment in the final solution falls within the comfortable range for measurement.

Example: 1 c.c. of blood was diluted to 100 c.c. with decinormal sodium hydroxide. To 1 c.c. of this solution was added 3 c.c. of the alkalin, then 1 c.c. of pyridine and, after mixing, a pinch of sodium hydrosulfite. In a 1 cm. cell the reading on the Hilger-Nutting photometer at 552 m $\mu$  was 0.573. The calibration for this instrument had been found to be 0.62. Hence:

$$\text{Hb. per c.c. of blood} = 0.573 \times 0.62 \times 500 = 177.6 \text{ mg. per c.c., i.e., 17.76 per cent}$$

The same result was obtained whether the determination was completed within five minutes or after allowing the mixture with alkali to stand for one hour and that with pyridine for a further half hour.

**B. DYSENTERIAE (Flexner), Experience With the Wilson and Blair Medium for, Bradshaw, D. B. Brit. M. J. 1: 181, 1942.**

Table of results with Wilson and Blair medium and those with MacConkey's Agar:

| Case No.  | Acute or Convalescent | No. of Flexner Colonies |                    | Remarks  |
|---|-----------------------|-------------------------|--------------------|--|
|   |                       | MacConkey Plate         | Wilson-Blair Plate |  |
| 240   | A                     | 1                       | 1                  | Pure culture of Flexner on Wilson-Blair plate  |
| 246   | A                     | +                       | +                  |  |
| 259   | C                     | Negative                | +                  |  |
| 262   | C                     | Negative                | +                  |  |
| 267   | A                     | 20                      | Uncountable        |  |
| 271   | C                     | 10                      | Uncountable        | Pure cultures of Flexner on Wilson-Blair plate |
| 272   | C                     | 2                       | 10                 |  |
| 275   | A                     | Negative                | Uncountable        |  |
| 284   | A                     | Negative                | Uncountable        | Little to choose between the two media         |
| 285   | A                     | Uncountable             | Uncountable        |  |
| 298   | C                     | Negative                | Uncountable        | Pure culture of Flexner on Wilson-Blair medium |
| 300   | C                     | Negative                | Uncountable        |  |
| 341   | C                     | 9                       | Negative           |  |
| 342   | C                     | 1                       | Negative           |  |
| 343   | C                     | 9                       | 100                |  |
| 353   | C                     | 1                       | 30                 |  |
| 372   | C                     | Negative                | Uncountable        |  |
| 383   | C                     | Negative                | 30                 |  |
| 384   | C                     | Negative                | 100                |  |
| 392   | C                     | Negative                | 10                 |  |
| Positive on both media: 8                                     |                       |                         |                    |  |
| Positive on Wilson and Blair's medium only: 10                |                       |                         |                    |  |
| Positive on MacConkey agar only: 2                            |                       |                         |                    |  |
| Total positive on Wilson and Blair's medium: 19 (90 per cent) |                       |                         |                    |  |
| Total positive on MacConkey's agar: 10 (50 per cent)          |                       |                         |                    |  |

**TAKATA-ARA REACTION, the Diagnostic Value of, Waugh, T. R., and McKenna, R. D. Am. J. M. Sc. 203: 722, 1942.**

Analysis of a series of 300 Takata-Ara reactions, carried out for the most part on patients who showed a hyperbilirubinemia or who gave reason to suspect the presence of hepatic disease, indicates that the test has considerable diagnostic value. It would appear that the difference of opinion which has arisen in the literature concerning its value is due to the use of various techniques and a lack of precision in reading the results. A means is described whereby more accurate results may be obtained.

The method used by the authors follows:

The test may be done on blood serum, plasma, or transudates such as ascitic fluid. Most of our results were obtained from oxalated plasma procured at the time of carrying out

a complete hemogram. Blood is usually taken for such tests in the morning, between 10 and 12 o'clock, to avoid any effect of digestive processes on the plasma. At least 1 c.c., free from hemolysis and cells, is required.

A series of 8 tubes, approximately 10 cm. in length and the inner bore of 1 cm., are placed in a rack. The tubes must, of course, be thoroughly clean. One cubic centimeter of 0.9 per cent sodium chloride is pipetted into each tube, and 1 c.c. of plasma is added to the first tube. This is mixed with the saline by shaking, and 1 c.c. of the mixture is withdrawn and placed in the second tube. By continuing in this manner and discarding 1 c.c. from the last tube a series of increasing dilutions (1:2 to 1:256) of plasma in saline is set up. To each tube, first 0.25 c.c. of a 10 per cent solution of sodium carbonate and then 0.15 c.c. of 0.5 per cent bichloride of mercury is added. Complete mixing is obtained by shaking after each solution has been placed in the tube. A slight precipitate, which disappears on shaking, may occur after addition of the mercury. The tubes are then allowed to stand at room temperature for twenty-four hours.

At the end of this interval the result of the test is read by estimating the amount of precipitate in each tube. This is expressed as 0, T (trace), 1, 2, 3, or 4. Precipitates classed as "4" occupy approximately the lower third of the solution in the tube. In doubtful cases, accurate reading of the amount of precipitate may be accomplished by thoroughly shaking up the tube and filling a Wintrobe sedimentation tube to the 10 cm. mark with a portion of the contents. The Wintrobe tube is then centrifugalized at high speed for fifteen minutes. Less than 1 mm. of whitish sediment in the bottom of the tube is classed as a trace (T), 1 to 2 mm. as "1," 2 to 3 mm. as "2," and so on. Any amount over 4 mm. is designated "4."

The Takata-Ara reaction is positive when two adjacent tubes show a grade "4" degree of precipitate and the next tube is at least a grade "3," for example (00T2441) or (00134421). Plus-minus reactions are reported whenever the criteria for the positive reaction are not reached, but at least two tubes show a grade "3" sediment, for example (00T12431), (0001331), or (000TT332). All reactions of lower degree are negative as (000TT231) or (000TT1TT). It would appear that accurate reading of the reaction is extremely important.

**C. DIPHTHERIAE, Further Studies on the Serological Classification of, Huang, C. H.**  
*Am. J. Hyg.* 35: 335, 1942.

Eight serologic types and an unidentified remainder have been found among the diphtheria bacilli isolated from the throats of 268 clinical patients with diphtheria.

Avirulent organisms, besides the virulent ones, were occasionally found in diphtheria patients in the acute and convalescent stages. In no instance was an avirulent organism found which was at the same time agglutinable by our S-type antisera, affording, therefore, no evidence for the transformation of a virulent diphtheria organism into an avirulent one in the throat of diphtheria patients.

The failure to find an avirulent organism, which is at the same time agglutinable by S-type antisera in acute and convalescent cases, together with the consistent finding of virulence in all the diphtheria organisms which belonged serologically to the author's S types, suggested the possibility of using the serologic agglutination as an indirect, but more convenient, means of determining the virulence of organisms in acute and convalescent cases.

Laryngeal diphtheria is less frequently observed in patients who were infected by type D<sub>1</sub> organisms than with other types of organisms. It happened that type D<sub>1</sub> is the only type of diphtheria organisms which possesses both gravis and intermedius strains.

The prevalence of types of organisms varies in different years.

Simultaneous and repeated swabs from the throat of the same patient practically always revealed the same type of organism. Only two patients were found to be infected by two different types of virulent organisms at the same time.

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## *PROGRESS*

### VARIATIONS IN SERUM MAGNESIUM IN HEALTH AND DISEASE: A REVIEW\*

VICTOR G. HACRY, M.D., PHILADELPHIA, PA.

#### INTRODUCTION

UNTIL 1932, when Kruse and his co-workers<sup>1</sup> demonstrated that magnesium is essential for the maintenance of life, very little physiologic significance was attached to it. In recent years, however, much more attention has been given to this element, particularly in regard to its variation in diseased states. It is unfortunate that such abundant researches on the biological significance of calcium should have been performed with an almost total omission of concomitant magnesium studies. This is all the more unfortunate since we now appreciate the close physiologic relationship which exists between calcium and magnesium.

The literature abounds with evidence that a derangement in calcium metabolism is either the cause or the effect of various clinical entities. Such information could have been obtained only by virtue of a great many studies made by the clinical chemists. Obviously the same must be done before any clinical significance can be attached to magnesium. A quotation from a recent editorial on "Magnesium in Nutrition"<sup>2</sup> adequately summarizes the present status of the magnesium problem. . . . "So little is known of the function of magnesium in the organism that clinically observable abnormalities in man cannot at present be said with certainty to be due to magnesium deficiency or to a disorder of magnesium metabolism. The systematic study of magnesium metabolism by accurate analytical and experimental methods is little more than begun. Future investigations may be expected to add considerably to our knowledge of this problem."

\*From the Department of Pharmacology, the Jefferson Medical College of Philadelphia.  
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Our attempt in this paper has been to review and to evaluate the scattered literature relative to the changes in blood magnesium in various diseases. The material is arranged, in so far as is possible, under the various clinical entities.

No definite conclusions can be drawn in most instances as to whether or not a hypomagnesemia or hypermagnesemia exists in a particular disease. Indeed, in some instances diametrically opposite results are reported by the various investigators. We can only report the facts as they are found, make criticisms where they are warranted, and point out certain trends if sufficient data existed to do so. It is hoped that this paper may serve to correlate, at least to some degree, the literature on this subject, and to stimulate further investigations on this vital and interesting subject.

#### NORMAL BLOOD VALUES

There is some disagreement in the literature as to what should be considered the serum magnesium level of normal persons. This discrepancy is due partly to the fact that the investigators used different methods for the determination of this element, and partly because some<sup>19</sup> considered results obtained from hospital patients as being normal. Obviously, such persons should not be considered as being normal, even if they do not have any apparent metabolic disturbances. The concentration of normal serum magnesium as determined by 18 different authors and the methods employed by each are shown in Table I.

TABLE I  
CONCENTRATION OF SERUM MAGNESIUM IN NORMAL PERSONS

| AUTHORS                                | PRECIPITATING AGENT | METHOD       | RANGE OF VALUE | MEAN |
|--|---------------------|--------------|----------------|------|
| Briggs <sup>3</sup>                    | Phosphate           | Colorimetric | 2.23-2.50      |      |
| Kramer and Tisdall <sup>4</sup>        | Phosphate           | Colorimetric | 1.80-2.30      | 2.10 |
| Bogert and Plass <sup>5</sup>          | Phosphate           | Colorimetric | 1.90-2.70      | 2.30 |
| Watchorn and McCance <sup>6</sup>      | Phosphate           | Colorimetric | 2.30-2.66      | 2.48 |
| Becher <sup>7</sup>                    | Phosphate           | Colorimetric | 1.80-2.30      |      |
| Wacker and Fahrig <sup>8</sup>         | Phosphate           | Colorimetric | 2.00-2.97      | 2.40 |
| Cope <sup>9</sup>                      | Phosphate           | Colorimetric | 1.82-2.63      | 2.06 |
| Walker and Walker <sup>10</sup>        | Phosphate           | Colorimetric | 1.60-3.00      | 2.20 |
| Brookfield <sup>11</sup>               | Phosphate           | Colorimetric | 1.89-2.19      | 2.04 |
| Bomskov <sup>12</sup>                  | Hydroxyquinoline    | Bromination  | 1.70-2.60      |      |
| Greenberg and associates <sup>13</sup> | Hydroxyquinoline    | Bromination  | 2.00-3.66      | 2.74 |
| Velluz and Velluz <sup>14</sup>        | Hydroxyquinoline    | Bromination  | 1.60-2.40      | 2.00 |
| Raices <sup>15</sup>                   | Hydroxyquinoline    | Bromination  | 1.69-3.00      | 2.44 |
| Hoffman <sup>16</sup>                  | Hydroxyquinoline    | Colorimetric | 1.90-2.50      | 2.18 |
| Hirschfelder and Haury <sup>17</sup>   | Titan yellow*       | Colorimetric | 1.80-2.40      | 2.11 |
| Haury <sup>18</sup>                    | Titan yellow        | Colorimetric | 1.70-3.10      | 2.33 |
| Bernstein and Simkins <sup>19</sup>    | Titan yellow        | Colorimetric | 1.23-3.54      |      |
| Wolf <sup>20*</sup>                    | Titan yellow        | Colorimetric | 2.90-4.00      | 3.61 |

\*For whole blood.

As seen from this table, the mean serum magnesium value of all the authors lies in the neighborhood of 2.4 mg. per 100 c.c. This is in close agreement to the value for serum magnesium of normal persons, as found by Tibbetts and Aub,<sup>50</sup> and corresponds closely to the mean value (2.33 mg.) found in normal medical students, as recently reported by Haury.<sup>18</sup> With the exception of the figures given by Greenberg, Lucia, Mackey, and Tufts,<sup>13</sup> and Bernstein and Simkins,<sup>19</sup> the serum magnesium of normal persons seems to lie between the lower and upper limits of 1.7 and 3.0 mg. per 100 c.c., respectively. Inasmuch as Wolf<sup>20</sup>



determined the magnesium content of whole blood, and since Greenberg and others<sup>13</sup> noted the fact that the red blood corpuscles contain from 5 to 9 mg. magnesium per 100 c.c., we would expect Wolf's figures to be higher than those obtained by other investigators using blood serum or plasma alone. In his review Shohl<sup>21</sup> states that the serum magnesium of normal persons lies somewhere between 1.0 and 3.0 mg. We believe, from our work and from the work of others, that the normal range is from 1.7 to 3.0 mg. per 100 c.c. of blood plasma or serum.

#### RENAL DISORDERS

Probably the first indication of a disturbance in magnesium metabolism was noted in cases of renal inadequacy. In 1923 Salvesen and Linder<sup>22</sup> reported a series of 15 cases in which the serum magnesium varied from 1.5 to 4.6 mg. per 100 c.c. of serum. In the same year Denis and Hobson<sup>23</sup> reported normal magnesium and potassium values in a series of 19 nephritic patients. They state, however, that the serum calcium was below normal in 5 cases. In view of the inverse relationship which exists between magnesium and calcium, i.e., as the serum magnesium increases the calcium decreases, and vice versa,<sup>11, 24-25</sup> one would be inclined to suspect an elevated serum magnesium in some of the patients studied by Denis and Hobson. In more recent years evidence has accumulated which seems to indicate that retention of magnesium is a common occurrence in cases of renal insufficiency.

In 1932 Beecher and Hamann<sup>26</sup> demonstrated that patients with diseased kidneys but with normal renal function had a normal serum magnesium (2.07 mg.). Patients showing a renal insufficiency, however, had a definitely elevated serum magnesium (3.49 mg. per 100 c.c.). In the same year Watchorn and McCane<sup>6</sup> reported 8 cases of uremia in which the serum magnesium ranged from 3.07 to 10.34 mg. per 100 c.c. Two years later Hirschfelder and Haury<sup>20</sup> demonstrated that in nephrectomized animals and in animals with injured kidneys, ingestion of magnesium sulfate caused a rapid increase in plasma magnesium, even to the point of coma. They suggested at that time that "uremic" coma may actually be produced or at least abetted by purgation with magnesium salts. It was pointed out also at that time<sup>21</sup> that a retention of magnesium was a common finding in uremic patients. Numerous papers appeared during the past few years confirming the foregoing findings.<sup>10, 11, 15, 32, 33</sup>

Occasionally a hypomagnesemia may be associated with renal insufficiency. In 1934 Hirschfelder and Haury<sup>21</sup> reported 4 cases of glomerulonephritis in which the serum magnesium was definitely below normal (0.9 to 1.31 mg. per 100 c.c.). In each of these cases either muscular twitchings or convulsions were present. This is of particular interest, since the publication of a report by Kruse, Orent, and McCollum,<sup>1</sup> in which they showed that animals raised on a magnesium-deficient diet have a tendency to become irritable and eventually have severe convulsive seizures. A few years later, Hoobler, Kruse, and McCollum<sup>24</sup> showed that this form of tetany is definitely due to a hypomagnesemia. It now becomes apparent that at least some of the so-called idiopathic tetanias, which cannot be explained on the basis of a low blood calcium, might be accounted for on the basis of a low blood magnesium. Perhaps the case of uremic convulsions described and treated with magnesium sulfate by Watson<sup>25</sup> was actually due to a concomitant hypomagnesemia.

On the grounds of Loeb's<sup>36</sup> pure physiologic work relative to the influence of ions on irritability, we could reasonably conclude that tetany can be due to a deficiency of serum magnesium. From his work we may set up the following relationship:  $\frac{\text{Na}^+ + \text{K}^+ + \text{OH}^-}{\text{Ca}^{++} + \text{Mg}^{++} + \text{H}^+}$ , as determining the state of irritability. An increase in any of the ions in the numerator, or a reduction of any of the ions in the denominator, will increase the irritability. From this scheme it becomes apparent that a reduction of magnesium is at least one possible factor in the etiology of hyperirritability.

As yet no human pathology has been demonstrated which can be attributed with certainty to a magnesium deficiency. Moreover, since Sherman<sup>37</sup> has shown that the ordinary diet contains more than enough magnesium to meet the daily requirement, it is questionable if a magnesium deficiency ever exists spontaneously in man. On the other hand, in animals we find a disease known as "grass staggers," the symptoms of which are strikingly similar to those seen in experimental magnesium tetany. Sjollesma and Seckles,<sup>38, 39</sup> and Metzger,<sup>40</sup> made an extensive study of this form of tetany. They found that the animals become nervous and restless, develop an unsteady gait, and finally die in violent convulsions. Blood chemistry studies in this disease show that the calcium and phosphorus levels are usually low, and that the magnesium is always low. Hopkirk<sup>41</sup> and Cunningham<sup>42</sup> confirmed these findings, and further demonstrated that the symptoms noted in these animals could be overcome by increasing the blood magnesium. It is interesting to note in this connection that Duncan, Huffman, and Robinson<sup>43</sup> produced an experimental tetany in 20 calves fed on whole milk or milk supplemented by iron, copper, manganese, silicon, aluminum, and cod-liver oil. Five of the animals which died in tetany had a normal blood plasma, calcium, and phosphorus, but a low blood plasma magnesium value. The tetanies in the calves due to a low blood magnesium were indistinguishable from those due to a low blood calcium. These could be differentiated only by determining the amounts of calcium and magnesium in the blood. The mean plasma magnesium value of the 5 calves (44 determinations) which died in tetany was 1.6 mg., as compared with the mean value of 2.41 mg. per 100 c.c. in 107 normal calves.<sup>44</sup> McGhee<sup>45</sup> showed that canine hysteria is due to a deficiency in blood magnesium rather than calcium.

We must also consider the possibility that some endocrine organ may control or at least influence magnesium metabolism. This possibility does not seem too hypothetical if one reviews the relationship which exists between sodium and the adrenal glands, iodine and the thyroid gland, and calcium and the parathyroids. Indeed, Coppo and Frugoni<sup>46, 47</sup> believe that the primary action of the parathyroids is to decrease the blood magnesium and that the calcium rises secondarily to antagonize the magnesium. Whether or not hypermagnesemia or hypomagnesemia is due to some endocrine dysfunction must be answered by future investigations.

Other pathologic conditions often associated with a hypomagnesemia will be discussed later under Endocrine Disturbances, Epilepsy, Carcinoma, and Asthma.

## DISEASES OF THE CIRCULATORY SYSTEM

Only fragmentary reports have been made on the serum magnesium value in circulatory diseases. In 1923 Weil, Guillaumin, and Weismann-Netter<sup>18</sup> observed an increase in the serum magnesium values of a few cases of essential hypertension. A few years later Blumgarten and Rohdenburg<sup>19</sup> reported 3 patients with arteriosclerosis and one with myocarditis having a high blood magnesium. Unfortunately, their results, obtained on whole blood, are difficult to compare with those of other investigators using serum or plasma. Wacker and Fahrig,<sup>5</sup> Walker and Walker,<sup>10</sup> and Bernstein and Simkins<sup>19</sup> demonstrated only a slight increase in serum magnesium in hypertensive cases not complicated with severe kidney damage. Beeher and Hamann<sup>20</sup> showed that the serum magnesium is nearly double the normal value in those patients having hypertension associated with kidney disease. Assuming that all patients with a severe hypertension have also an abnormal kidney function, one would expect at least some elevation in serum magnesium in nearly all cases suffering with this disease.

## ENDOCRINE DISTURBANCES

(a) *Diabetes Mellitus*.—Tibbetts and Anb have shown<sup>20</sup> that the ingestion of an acid-producing salt ( $\text{NH}_4\text{Cl}$ ) causes a negative calcium balance. Their studies suggest further that magnesium may be used in the body as a substitute for calcium base, the excess usually appearing in the urine. From what we already know of the inverse relationship between calcium and magnesium, we would expect the serum magnesium in such conditions to be definitely elevated. We could speculate then that in cases of diabetic acidosis, as well as in acidosis due to any other cause, a negative calcium balance should exist with a simultaneous elevation in serum magnesium. Our factual information in this direction is entirely inadequate. Brookfield,<sup>11</sup> and Watchorn and McCance,<sup>6</sup> reported three patients with diabetic coma as having a high serum magnesium, whereas a number of similar cases not in coma were said to have a normal serum magnesium. Three of the 7 cases reported by Blumgarten and Rohdenburg<sup>19</sup> were found to have a high and two were found to have a low blood magnesium. None of their patients were in coma.

(b) *Diseases of the Thyroid*.—Blumgarten and Rohdenburg<sup>19</sup> reported 45 patients with goiter on whom blood magnesium determinations were made. In these 15 showed a slight hypomagnesemia and 5 showed a slight hypermagnesemia. Watchorn and McCance,<sup>6</sup> and Beeher and Hamann,<sup>20</sup> found normal values in the few cases they reported.

Recently Soffer and co-workers<sup>21a</sup> made an interesting study on the state of the serum magnesium in 31 patients with hyperthyroidism. They found the total serum magnesium in these patients to vary between 1.85 and 2.96, with an average of 2.44 mg. per cent. The total serum magnesium of 14 normal persons varied between 2.12 and 2.76, with an average of 2.52 mg. per cent. However, a striking difference in the ultrafiltrable magnesium was noted. In normal persons this varied between 77.9 and 96.9, with an average of 85.5 per cent. In the patients with hyperthyroidism the ultrafiltrable magnesium was greatly diminished, varying between 38.4 and 78.5, with an average of only 64 per cent. In a

later paper Soffer and associates<sup>51b</sup> made a similar study on an additional 50 patients with hyperthyroidism. Essentially the same results were obtained. In addition they reported that in patients with myxedema, as well as in totally thyroidectomized dogs, the ultrafiltrable serum magnesium is extremely high and frequently is elevated as much as 100 per cent. Since the ultrafiltrable fraction is probably the physiologically important fraction, we could deduce from these results that in hyperthyroidism an actual state of hypomagnesemia exists and that myxedema patients are in a state of hypermagnesemia. This may help to explain the hyperirritability and hypoirritability so commonly associated with Graves' disease and myxedema, respectively. It certainly stresses the importance of determining the amount of physiologically active (diffusible) fraction of this ion before an actual state of hypermagnesemia or hypomagnesemia can be said to exist.

(e) *Diseases of the Parathyroids.*—From the dramatic influence that the parathyroids have on calcium metabolism, we might expect them to have a similar effect on magnesium metabolism. The immediate effect of parathyroid hormone on serum magnesium has been studied in dogs by Scholtz<sup>52</sup> and by Greenburg and Maekey.<sup>53</sup> Their investigations showed that the serum magnesium increased slightly in two to four hours following the injection of the parathyroid hormone. This disappears long before the calcium has reached its maximum value. Bulger and Gausmann<sup>54</sup> noticed that prolonged administration of parathormone to dogs resulted in no striking alterations in serum magnesium. Following the administration of 200 units of parathormone in human beings, Melli and Karadimova<sup>55</sup> observed that the serum magnesium increased during the first hour, reached the maximum at the third hour, and fell to normal at the fourth hour. Serum calcium, on the other hand, began to rise only after the fourth hour. Coppo and Pisa<sup>56</sup> found that one unit of parathormone per day administered to a rat produced an increase in the blood serum calcium and a reduction in the blood serum magnesium. The authors conclude that their analyses furnish an important contribution to the theory that assumes a direct relationship between the function of the parathyroids and the biochemistry of magnesium.<sup>46, 47</sup> Cantarow, Haury, and Whitbeck<sup>57</sup> found that large doses of parathormone injected intramuscularly in dogs produced a slight decrease in serum magnesium in the majority of their experiments. In a human subject Watchorn and McCance<sup>6</sup> also found that intramuscular injections of the hormone produced a slight but definite fall in the serum magnesium.

Denis and Talbot,<sup>58</sup> Bulger and Gausmann,<sup>54</sup> and also Hartog and Muller<sup>32</sup> found a hypomagnesemia in clinical cases of parathyroid tetany. The few serum magnesium studies made in patients with hyperparathyroidism reported by Tibbetts and Aub<sup>59</sup> indicate that a relatively normal serum magnesium prevails in spite of the hypercalcemia. Future investigations in this field should prove most interesting.

(d) *Miscellaneous Endocrine Disturbances.*—Practically nothing is known of the status of serum magnesium in other types of endocrine disturbances. Blumgarten and Rohdenburg<sup>49</sup> found the whole blood magnesium to be within the normal limits in all cases of pituitary dysfunction. Tibbetts and Aub,<sup>61</sup> and

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# CLINICAL AND EXPERIMENTAL

## THE PHARMACOLOGY OF $\beta$ (3,5 DI-iodo-4-HYDROXYPHENYL) A PHENYL PROPIONIC ACID\*

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**B**ILISELECTAN† has been used in recent years in Germany for gall bladder visualization.<sup>1-5</sup> Superiority over the radio-opaque phenolphthalein dyes for outlining the gall bladder is claimed because dense shadows may be obtained after oral administration with unpleasant symptoms appearing only rarely. A systematic experimental study of biliselectan has not been published. Inasmuch as the drug may be a useful one the present investigation was undertaken to determine its actions and toxicity.

### PHYSICAL AND CHEMICAL PROPERTIES

Biliselectan is  $\beta$ (3,5 diiodo-4-hydroxyphenyl) $\alpha$  phenyl propionic acid. It is a faintly cream-colored powder, insoluble in water, and practically tasteless. It dissolves in alkali, presumably in the form of the sodium salt, when sodium hydroxide is used. Solutions which were used, 2.5 and 5.0 per cent, made up with the minimum amount of sodium hydroxide necessary to bring about complete solution without heat, have a pH of 11.5 or 11.6. These solutions are yellow and clear. A 5.0 per cent solution, kept for forty-eight hours at room temperature in the light, did not show free iodine by the starch test.

### EXPERIMENTAL PROCEDURE

Cats were used in several types of experiments, acute and chronic, to study toxicity. The behavior, appearance, and weight of the animals were recorded. Examinations were made for evidence of changes in blood nonprotein nitrogen, red blood cell count, red blood cell fragility, and clotting time. Phenolsulphonphthalein excretion tests were made, and the urine was examined for albumin and reducing substances. These observations were made prior to and for a varying period after the administration of the drug. In the course of the observations on 33 animals, 20 red cell counts, 30 red cell fragility determinations, 20 clotting time determinations, 50 nonprotein nitrogen determinations, 40 phenolsulphonphthalein excretion tests, and 85 urinalyses were made. Most of these tests were made in a series of 12 cats used in chronic experiments.

\*From the Department of Pharmacology Cornell University Medical College, New York. Received for publication, February 16, 1942.

†The original proprietary name, Biliselectan, will be used throughout the text, instead of the more cumbersome chemical name. The drug may be secured from the Schering Corporation, which now designate it Priodax.



At autopsy the organs of all animals were examined grossly, and in the case of the animals in the chronic experiments, the livers and kidneys were studied histologically.

All intravenous injections were made with a 2.5 or 5.0 per cent alkaline (pH 11.5 to 11.6) solution, while most of the oral doses were given as the powder in capsules.

TABLE I

EFFECTS OF INTRAVENOUS INJECTIONS OF BILISELECTAN IN UNANESTHETIZED ANIMALS

| CAT. NO. | TOTAL DOSE<br>INDIVIDUAL DOSE* | NUMBER OF INJECTIONS | TIME BETWEEN FIRST AND LAST INJECTION | CONVULSIONS | TIME TO DEATH AFTER FIRST INJECTION | REMARKS                                       |
|----------|--------------------------------|----------------------|---------------------------------------|-------------|-------------------------------------|---|
| 5        | 150                            | 1                    | 0                                     | Yes         | 1 minute                            |   |
| 6        | 285                            | 4                    | 4 days                                | No          | Did not die                         |   |
|          | 6.1/50/75/100                  |                      |                                       |             |                                     |   |
| 7        | 150<br>75-75                   | 2                    | 45 minutes                            | Yes         | 112 minutes                         |   |
| 8        | 200<br>100/100                 | 2                    | 26 hours                              | Yes         | 27 hours                            | Small pulmonary hemorrhages                   |
| 9        | 100                            | 1                    | 0                                     | Yes         | 97 minutes                          |   |
| 10       | 100                            | 1                    | 0                                     | Yes         | 46 minutes                          |   |
| 11       | 500<br>100-150/250             | 3                    | 24 hours                              | Yes         | 25 hours                            |   |
| 12       | 250<br>100-150                 | 2                    | 21 minutes                            | Yes         | Between 12 and 24 hours             | Found dead in morning after apparent recovery |
| 13       | 250<br>100-150                 | 2                    | 27 minutes                            | Yes         | 31 minutes                          |   |
| 14       | 250<br>100-150                 | 2                    | 10 minutes                            | Yes         | 150 minutes                         |   |

\*The numbers underneath the total dose indicate the individual doses. Those doses separated by a dash (-) were given on the same day, whereas those doses separated by a line (/) were given on different days.

## RESULTS OF EXPERIMENTS

*Effect of pH.*—Since the intravenous injections of the alkaline solutions of biliselectan were followed by convulsions and other systemic effects, it was necessary to eliminate the alkali itself as a factor in the production of these effects. Therefore, two unanesthetized cats were injected with a solution of sodium hydroxide with a pH (11.5) the same as that of the biliselectan solutions used. Injections of this solution were without noticeable effect even when used in amounts 2.5 times that of biliselectan doses which produced violent convulsions.

*Effects of Intravenous Injections on Circulation and Respiration in Anesthetized Animals.*—The effect on cardiodynamics and respiration was studied in 4 cats which were anesthetized with sodium amytal and whose blood pressure and respiration were recorded on a kymograph.

Effects were produced which varied from animal to animal. For example, in one animal (Fig. 1) the first injection of 100 mg. per kilogram\* produced

\*All drugs were administered in relation to body weight, but for the purpose of convenience the expression "per kilogram" will not be repeated.

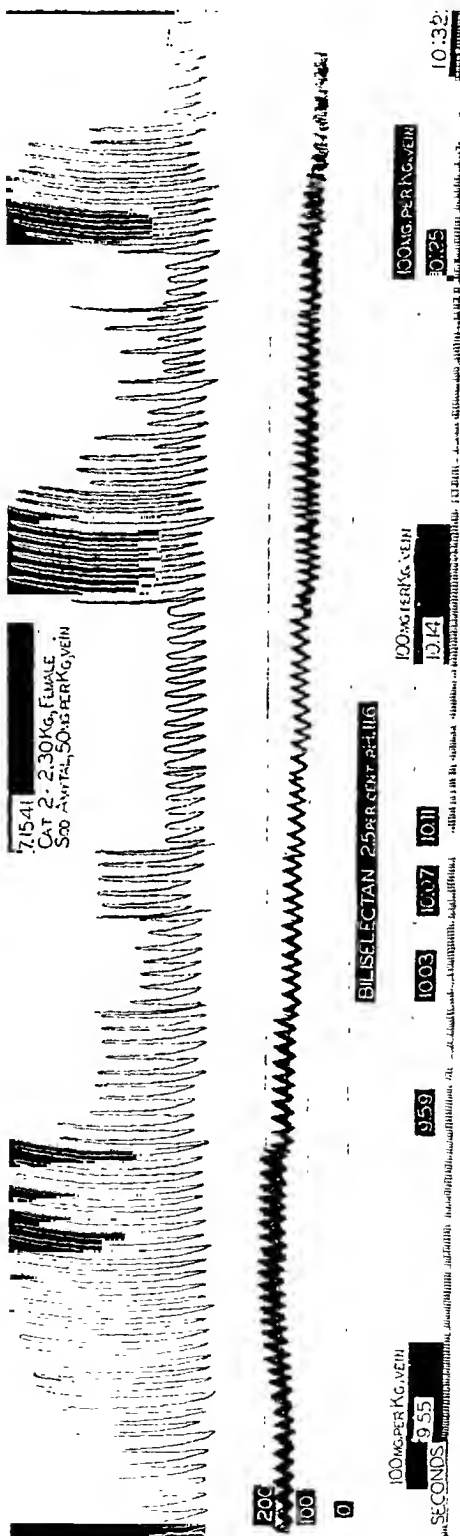


Fig. 1.—Respiratory stimulation produced by biliselectan. Respiration is shown in the upper curve; blood pressure and heart rate in the lower. The calibration lines for the blood pressure indicate millimeters of Hg. The next line in descending order indicates the period of injection, while the last indicates the time in seconds.

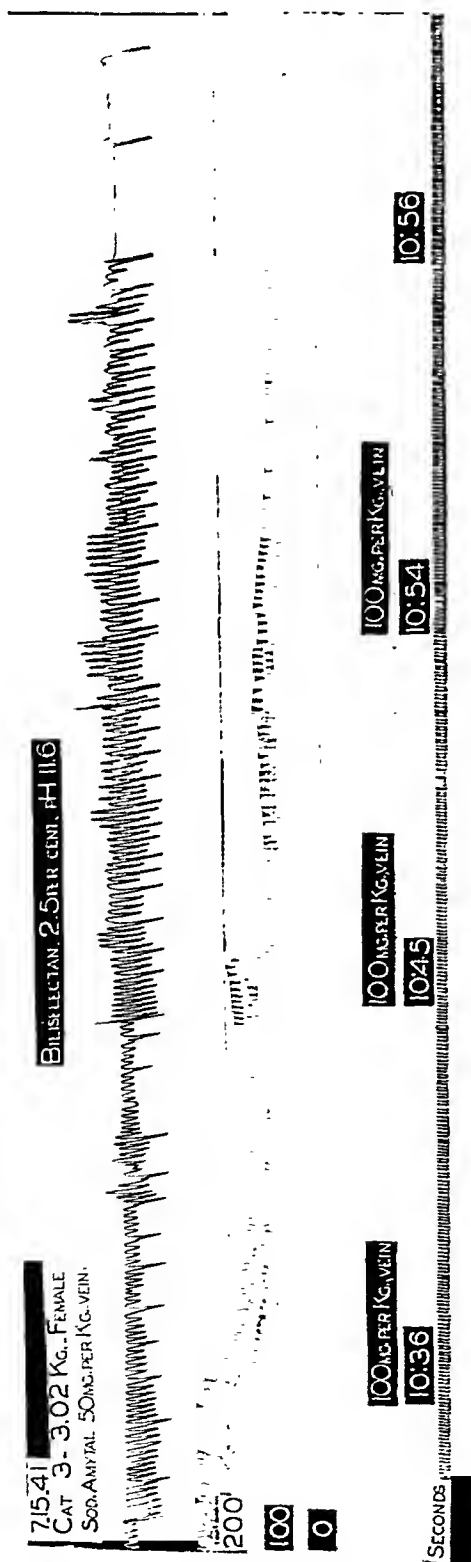


Fig. 2.—Depression of blood pressure, without respiratory depression, produced by doses similar to those shown in Fig. 1.

marked respiratory stimulation lasting almost five minutes. Subsequent similar doses produced progressively less stimulation. On the other hand, in another animal (Fig. 2) receiving the same doses of the same solution there was no respiratory stimulation, but rather a fall in blood pressure after each dose.

Fatal doses were followed by a fall in blood pressure and respiratory depression. In 2 cats the respiratory failure appeared first, the heart continuing to beat after the cessation of breathing. In the other 2 cats the cardiac and respiratory failure appeared simultaneously. In 3 of the 4 cats the terminal events occurred suddenly and without warning.

*Effect of Intravenous Injections in Unanesthetized Animals.*—In 10 unanesthetized cats the effects of intravenous injections were observed. The significant data are summarized in Table I.

*Lethal dose.* Single injections of 50, 60, and 75 mg. were not fatal. Injections of 100 mg. killed 2 of 8 cats. A single injection of 150 mg. killed one cat, while another died of the same dose given in two parts within forty-five minutes. Each of 3 cats died of 250 mg. given within thirty minutes in two doses, while one cat survived this dose.

*Convulsions.* Biliselectan is a convulsant agent similar to picrotoxin or camphor. It produces typical medullary convulsions with all the autonomic phenomena which are associated with it, salivary stimulation, respiratory stimulation and depression when doses of 100 mg. or more are administered. Doses of 50 and 60 mg. failed to induce convulsions, whereas only one of two injections of 75 mg. was followed by convulsions.

The convulsions were always immediate and violent, and without reflex hyperexcitability. Sometimes the convulsions would persist for as long as four to six hours, but neither the duration nor the intensity of the convulsions was an indication of whether the animal would survive a particular dose.

*Antidote.* The group of four experiments, already described, in which the blood pressure records were made, were performed with amytal anesthesia. In this group the drug proved to be fatal in divided doses of from 200 to 600 mg. without convulsions. The protective action of amytal against biliselectan was studied in another series of 4 cats in which single intravenous doses of from 200 to 350 mg. were administered after 50 mg. of sodium amytal. The two animals which received 200 mg. of biliselectan were protected against the fatal action as well as the convulsions, whereas the cats receiving 300 to 350 mg., respectively, died, although no convulsions were induced. It is to be assumed, therefore, that the fatal effects of biliselectan are only in part the result of convulsions, since with somewhat larger doses, namely, 300 mg., the animals die, even though convulsions are prevented. It is interesting to note that the large doses of biliselectan, through its stimulation of the higher centers, brought about fleeting signs of reversal of the narcotic action of amytal.

*Persistence of action.* The persistence of biliselectan action is relatively brief. Very little of a given dose appears to remain after twenty-four hours. For example, cat 6 received on successive days injections of 60, 50, 75, and 100 mg. of biliselectan. Each injection was followed by systemic effects: excitement, ataxia, depression, respiratory stimulation, yet the animal recovered

completely after each injection, even the last, although a total of 285 mg. had been administered.

*Tissue changes.* The post-mortem examination of the organs revealed abnormal findings in only one case. In that there were found minute hemorrhages in the lungs surrounded by small pink areolae. These may have been pin-point pulmonary emboli. Examination for the fragility of the red blood cells was made in 4 cats and no change was found.

TABLE II  
EFFECTS OF SINGLE ORAL DOSES OF BILISELECTAN

| CAT NO. | DOSE  | PERIOD OF OBSERVATION (DAYS) | INTERVAL BEFORE VOMITING         | IMPAIRED APPETITE, DURATION (DAYS) | ALBUMINURIA |                 | REMARKS   |
|---------|-------|------------------------------|----------------------------------|------------------------------------|-------------|-----------------|---|
|         |       |                              |                                  |                                    | DEGREE      | DURATION (DAYS) |   |
| 15      | 100   | 28                           | No vomiting                      | None                               | +           | 1               | Less than 10 per cent drug lost in vomiting. Diarrhea for one day                             |
| 16      | 250   | 28                           | 5¼ hours                         | 1                                  | ++          | 1               |   |
| 21      | 250   | 5                            | No vomiting                      | None                               | +           | 2               |   |
| 22      | 250   | 2                            | No vomiting                      | None                               | 0           |                 |   |
| 23      | 250   | 8                            | No vomiting                      | None                               | 0           |                 |   |
| 17      | 500   | 15                           | 20 minutes                       | 1                                  | +           | 6               | About 50 per cent drug lost in vomiting. Pregnant—aborted second day. Depressed for five days |
| 20      | 500   | 28                           | 3 hours                          | 1                                  | +           | 7               | Less than 10 per cent drug lost in vomiting. Hematuria and glycosuria for one day             |
| 24      | 500   | 6                            | 2½ hours                         | 6                                  | ++          | 6               | Nonprotein nitrogen rises to 127, cloudy swelling of tubules, marked depression               |
| 25      | 500   | 2                            | 1 hour*                          | 2                                  | +++         | 2               | Died on sixth day. Marked depression  |
| 26      | 500   | 6                            | 2½ hours*                        | 6                                  | ++          | 4 plus          |   |
| 18      | 1,000 | 28                           | 7 minutes                        | 2                                  | +           | 1               | About 75 per cent drug lost in vomiting. Diarrhea for one day, depressed for one day          |
| 19      | 1,000 | 8                            | No vomiting                      | 8                                  | +           | 1               | Depressed for eight days. Died of pneumonia   |
| 31      | 1,000 | 1                            | Vomiting prevented with morphine | 1                                  | +++         | 1               | Found dead morning after drug was administered  |
| 32      | 1,000 | 1                            |                                  | 1                                  | ?           | ?               | Found dead morning after drug was administered  |
| 33      | 1,000 | 28                           |                                  | 4                                  | ++++        | 5               | Glycosuria five days; moderate depression for seven days                                      |

\*In these animals there was no loss of the drug by vomiting, since there was no expulsion, only retching.

*Chronic Experiments—Oral Administration.*—The effect of a single oral dose of from 100 to 1,000 mg. was studied in 12 cats for a period of from two to twenty-eight days. The drug was administered usually in capsules after food had been withheld for twelve hours.

The effects are summarized in Table II. There were two deaths which could be ascribed to the biliselectan. Both animals received 500 mg. One died on the sixth day and the other was killed on the sixth day when it was moribund. Another cat which received 1,000 mg. died of a respiratory infection.

Albuminuria was produced in 10 of the 12 cats. It was present in all 7 cats which received 500 mg. or more and in 3 of the 5 cats which received 100 and 250 mg. The albuminuria was transient. It persisted from one to seven days. Glycosuria occurred in one cat, in which it lasted only one day.

Digestive disturbances were common. Vomiting was seen in 7 of the 12 cats; in 1 of 4 which received 250 mg., in all 5 which received 500 mg., and in 1 of 2 which received 1,000 mg. In two instances vomiting was noted seven and twenty minutes after the drug was administered. In the other cases it was delayed for from one to five hours. The appetite was impaired in all the cats which received 500 mg. or more and in one of 5 receiving less. The impairment of the appetite lasted only a day or two in most cats but persisted as long as six to eight days in 3 cats which died. Post-mortem examination did not reveal any evidence of intestinal irritation, but since the intravenous injections did not produce vomiting it was assumed that the digestive disturbances were produced by a local rather than a central action. The loss of biliselectan by vomiting was as indicated in the table, in most cases not sufficient to influence the results.

There was a varying degree of general depression in 5 cats (indisposition to move, lethargy) after doses of 500 mg. or more which usually lasted for from five to eight days.

The average weight of the 4 cats which were permitted to survive for twenty-eight days was 2.42 kg. as compared with 2.61 kg. when the experiment was started. Such a small loss of weight (10 per cent) for cats confined for a four-week experimental period cannot be attributed to the drug.

Biliselectan produced no change in the red cell count, red cell fragility, and clotting time of the blood. In only one cat was there a rise in the non-protein nitrogen level. None of the phenolsulphonphthalein excretion tests indicated a significant deviation from the control.

The post-mortem examinations and the histologic studies of the livers and kidneys were negative with the exception of one animal discussed below.

The two animals which were killed by the 500 mg. dose of biliselectan suffered from a persistent albuminuria, vomiting, depression, and a loss of appetite which became progressively more profound. One was found dead in a state of decomposition which precluded examination of blood or tissues. In the other the nonprotein nitrogen level in the blood had risen to 127 mg. per cent on the day of death. On the sixth day it appeared moribund and began to vomit again. It was killed in order to obtain satisfactory tissue for histologic study. In the gross examination of the organs there was no significant finding other than the pale kidneys and a small pneumonic patch in the right lung. On microscopic examination of the kidneys, cloudy swelling of the renal tubules was found.

In another series of chronic experiments the complicating factor of vomiting and expulsion of the drug was removed. Three cats were given morphine intravenously to prevent vomiting and a dose of 1,000 mg. of biliselectan orally. Two of the 3 cats were depressed and suffered complete loss of appetite within four hours, and were found dead the following morning. In one of these 2 animals albumin was found in the bladder urine and venous congestion and

parenchymatous degeneration of the tubules of the kidneys on microscopic examination, but no evidence of gastrointestinal irritation. The kidney was normal in the other and its bladder was empty when found so that no urine examination could be performed.

The third cat was slightly depressed for seven days with impairment of appetite for four days. Albumin and glucose were found in the urine for the first five days. There was no significant change in the blood nonprotein nitrogen at any time. After a week the animal appeared entirely normal and continued so for the duration of the experiment (twenty-eight days). On gross and microscopic examination of the organs at the end of this period no pathologic changes were found.

#### SUMMARY AND CONCLUSIONS

The pharmacology of biliselectan,  $\beta$  (3,5 di-iodo-4-hydroxyphenyl)  $\alpha$  phenyl propionic acid, a substance which has been used for gall bladder visualization, was studied in acute and chronic experiments in 33 cats.

By intravenous injection biliselectan acts as a medullary convulsant. Intravenous doses as small as 50 to 75 mg. per kilogram cause excitement with respiratory stimulation. Larger doses cause myoclonic convulsions with stimulation and secondary depression of the circulation and respiration. The intravenous LD 50 ranges about 150 mg. per kilogram. The convulsant action can be abolished by the barbiturate, sodium amytal. In that case, a somewhat larger dose proves fatal by vasomotor and respiratory paralysis without convulsions.

The persistence of action of the intravenous dose appears to be brief, of the order of twenty-four hours or less, as seen by the fact that little cumulation is in evidence when intravenous doses are repeated. However, very large doses by mouth may cause a form of injury which tends to be progressive, so that an animal may die six days after a large oral dose.

The drug is much less toxic by oral administration. By this route animals survive doses as high as 1,000 mg. per kilogram, suggesting that the absorption is very imperfect. Marked individual differences, however, occur. Thus, one animal, after 1,000 mg. per kilogram (vomiting prevented by morphine), showed impaired appetite and slight depression within a few hours after the dose, albuminuria the following day, all of these effects wearing off within seven days; another animal receiving 500 mg. per kilogram showed more severe gastrointestinal symptoms which increased progressively until the animal became moribund and died on the sixth day. Biliselectan given orally does not produce convulsions.

In a total of 15 chronic experiments the effects of single oral doses were observed for periods up to twenty-eight days. Doses of 250 mg. per kilogram or larger cause impaired appetite, nausea, and vomiting and general depression. The intensity of these effects increases with the dose. The emetic effect is due to local action in the gastrointestinal tract, since it does not occur after the intravenous injection. Albuminuria is an outstanding effect. It occurs with doses as low as 100 mg. per kilogram. It is transient, and the duration of this effect varies with the dose.

Doses of from 100 to 1,000 mg. per kilogram are without effect on the non-protein nitrogen level of the blood, the phenolsulphonphthalein excretion, the red blood cell count, the red blood cell fragility, and the blood clotting time, even though some of the larger doses prove fatal. There are no histologic changes in the kidneys. There were two exceptions, one animal which became depressed after 500 mg. per kilogram, and a nonprotein nitrogen determination during the moribund state (on the day of death) showed a very high level, namely, 127 mg. per cent, and another was found dead the morning after 1,000 mg. In these animals the kidney tubules showed cloudy swelling. Since 1,000 mg. doses failed to cause this effect in 4 other animals, the possibility remains that the blood and renal changes in these cases may have been related to the profound depression of the circulation.

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## THE OCCURRENCE OF *TRICHINELLA SPIRALIS* LARVAE IN TISSUES OTHER THAN SKELETAL MUSCLES\*

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WHILE the larvae of *Trichinella spiralis* develop primarily in the skeletal muscles, there have been reported in the medical literature about 20 fatal cases of trichinosis in which other organs and tissues have been involved. In such cases the myocardium<sup>6</sup> and, to a lesser extent, the central nervous system<sup>4</sup> have been most frequently injured, but larvae have also been reported from the pancreas, kidney, liver, gall bladder, smooth muscles of the intestine, and tissues of the respiratory tract. In addition to the fatal cases there has been reported a far greater number of nonfatal cases with apparent involvement of various organs and systems other than the skeletal muscles. In this connection, we have been particularly impressed by the number of patients who have shown temporary loss of reflexes, and by the number whose spinal fluids have been found positive for larvae.<sup>1, 5</sup>

From our knowledge of the life history of *T. spiralis*, it seems altogether likely that the so-called migrating larvae are carried passively in the blood stream to all parts of the body. Since the larvae have a diameter in cross sec-

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tion of about  $6\mu$ , i.e., less than that of an erythrocyte, it is possible that they are able to negotiate the various capillary beds and to continue uninterruptedly in the blood stream until they reach the skeletal muscles, where they escape from the vascular system, penetrate the muscle fibers, and encyst. A second possibility, however, is that they are carried in the blood to all organs and tissues, which they indiscriminately invade, but in none of which, except striated muscle, are they able to develop. Weight is given to this alternative by the cases referred to above, and by reports such as that of Dunlop and Weller<sup>3</sup> in which it was demonstrated that larvae invade the myocardium of rats and are destroyed in situ. The purpose of this study was to ascertain as precisely as possible the extent to which migrating trichina larvae are found in tissues other than skeletal muscles.

#### MATERIALS AND METHODS

Young mice (5 to 6 weeks old) were infected by esophageal tube with 450 one- to two-month-old trichina larvae freshly isolated from rat muscle by artificial digestion (1.0 per cent pepsin and 0.7 per cent hydrochloric acid). The mice were sacrificed four to fifteen days later and exsanguinated. The blood was collected in sodium citrate, mixed with sufficient distilled water to lyse the cells, and centrifuged, and the sediment was examined for larvae. In attempting to recover the larvae from the tissues of the experimental mice, the foregoing digestion method and the use of other digesting agents failed because the migrating larvae were too young to withstand digestion. Likewise, a modified Baermann apparatus<sup>2</sup> maintained at various temperatures was unsuccessful because the larvae did not migrate out of the macerated tissues and into the funnel. Most of the results reported herein were obtained by direct microscopic examination with 16 mm. objective and 10 $\times$  oculars, of tissues teased apart and pressed between slides. Attempts at supravital staining in such preparations were abandoned after four commonly used stains (Janus green B, sudan III, Bismarck brown, and methylene blue) failed to facilitate the finding of larvae. The results obtained by direct examination were supplemented by the even more laborious study of serial sections of various tissues.

#### RESULTS

About 200 slides of serially sectioned tissues of 22 mice were examined. Larvae were seen in the skeletal muscles of animals killed as early as the sixth day after infection. In addition, a few inflammatory foci suggestive of the presence of worms were seen in the myocardium of mice twelve to fourteen days after infection but, since this has been adequately described by Dunlop and Weller,<sup>3</sup> no effort was made to pursue the subject. The direct examination of teased and pressed tissues was on the whole more satisfactory. Unbroken living larvae could be readily seen and identified even in the very young stages, and an entire organ could be examined effectively in much less time than was required for examination of serial sections of equivalent amounts of tissue. Even by this process, however, only 31 larvae were found in addition to those seen in the skeletal muscles and lysed blood (Table I). Of these 31 larvae 24 were recovered from the myocardium. It will be recalled that the methods used did not

permit our determining whether such larvae were free in the tissues or whether they were confined within the vascular system of the organs involved.

In experiments designed to determine whether or not the very young larvae had the capacity of directional movement and could, therefore, respond actively to stimuli, larvae freshly isolated from the uteri of six-day-old female worms were placed on warm, moist slides. Such larvae were observed in more or less continuous motion for several hours, but they never moved from the spot in which they were placed. The addition of freshly teased muscle fibers and of small amounts of other tissues failed to induce directional movement.

TABLE I

OCURRENCE OF *T. spiralis* IN ORGANS OF MICE INFECTED WITH 450 LARVAE

| MOUSE NO. | DAYS AFTER INFECTION | BLOOD (LARVAE/C.C.) | DIAPHRAGM | HEART WALL | LUNG (ONE) | LIVER (ONE LOBE) | KIDNEY (ONE) | TESTIS (ONE) | BRAIN (ONE-HALF) | SPLEEN |
|-----------|----------------------|---------------------|-----------|------------|------------|------------------|--------------|--------------|------------------|--------|
| 501       | 4                    | 0                   | 0         | 0          | 0          | 0                | 0            | 0            | 0                | -      |
| 502       | 5                    | 0                   | 0         | 0          | 0          | 0                | 0            | -            | 0                | -      |
| 502a      | 5                    | 1-2                 | 0         | 0          | 0          | 0                | 0            | 0            | 0                | -      |
| 503       | 6                    | -                   | +         | 0          | 0          | 0                | 0            | 0            | 0                | 0      |
| 503a      | 6                    | 10                  | +         | 0          | 0          | 0                | 0            | 0            | 0                | 0      |
| 504       | 7                    | 20                  | ++++      | 2          | 0          | 0                | 0            | 0            | 0                | 0      |
| 504a      | 7                    | 10                  | ++++      | 1          | 0          | 0                | 1            | 0            | 0                | 0      |
| 505       | 9                    | +                   | ++++      | 6          | 0          | 2                | 0            | 0            | 0                | 0      |
| 505a      | 9                    | +                   | ++++      | 0          | 0          | 0                | 0            | 0            | 0                | 0      |
| 506       | 10                   | 40                  | ++++      | 6          | 0          | 0                | 0            | 0            | 0                | 0      |
| 506a      | 10                   | 80                  | ++++      | 0          | 0          | 0                | 0            | 0            | 0                | 0      |
| 507       | 11                   | +                   | ++++      | 4          | 0          | 0                | 1            | 0            | 0                | 0      |
| 507a      | 11                   | 45                  | ++++      | 0          | 0          | 0                | 0            | 0            | 0                | 0      |
| 508       | 13                   | 50                  | ++++      | 2          | 0          | 2                | 0            | 0            | 0                | 0      |
| 508a      | 13                   | 100                 | ++++      | 3          | 1          | 0                | 0            | 0            | 0                | 0      |
| 509       | 14                   | 70                  | ++++      | 0          | 0          | 0                | 0            | -            | 0                | -      |
| 510       | 15                   | 80                  | ++++      | 0          | 0          | 0                | 0            | -            | 0                | -      |

## DISCUSSION

It becomes evident from the foregoing experiments that no great number of *T. spiralis* larvae normally penetrates tissues other than striated muscle in white mice. Despite the afore-mentioned impossibility of determining whether the few larvae that were observed in other tissues had left the vascular system or not, it is interesting to consider the ratio of such larvae to the total number. Since the mice were killed early in the course of their infections, we have no direct measure of the number of larvae which would have developed. In other experiments, however, it was found (Mauss, MSS.) that an average of 26,000 larvae was produced in 13 white mice receiving infective doses of 210 to 1,320 larvae. Using this figure as at least a crude measure of the infections in question, we find that 31 larvae were recovered from organs and tissues other than the striated muscle (or lysed blood) out of a possible final total of 312,000 larvae in the 12 animals killed between the seventh and fifteenth day. This is roughly one in 10,000.

Since it seems unlikely that the situation could be materially different in man than in the white mouse, it would appear that there is usually relatively little trauma in tissues and organs other than skeletal muscles in human trichinosis. Temporary neurologic disturbances or other clinical manifestations may

result from the occasional larvae entering the nervous system or other tissues. In the fatal cases of trichinosis the much heavier infection could increase the likelihood of damage to various organs and tissues without any change in the proportion of larvae invading such tissues. The possibility should not be overlooked, however, that some abnormal distribution of larvae may have operated in those heavier infections of the reported fatal cases.

#### SUMMARY

Thirty-one migrating larvae were recovered from the carefully examined tissues and organs other than muscles and blood of 12 white mice killed seven to fifteen days after infection with *T. spiralis*. It is estimated that approximately 10,000 times as many larvae are found in the skeletal muscles of mice receiving comparable infections. Apparently, then, invasion of organs and tissues other than skeletal muscles is of minor significance except in peculiarly heavy infections.

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### THE TREATMENT OF ACUTE MERCURY POISONING WITH SODIUM FORMALDEHYDE SULFOXYLATE WITH A REVIEW OF TWENTY CASES\*

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THERE are two fundamental concepts in the formulation of any method of therapy for acute mercury poisoning. The first is the removal of the unabsorbed mercury as soon as possible in order to prevent further absorption. The second is the removal of the absorbed mercury rapidly with the least toxic effect to the organism.

In cases of oral ingestion the first of these concepts is best carried out by simple gastric lavage, using large quantities of fluid. Thus, any unabsorbed mercury is washed out. If such a procedure has been preceded by vomiting, so much the better, and, when immediate lavage cannot be instituted, fluid should be given after which vomiting should be induced.

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However, even when vomiting is prompt and lavage is done soon after ingestion, absorption of the mercury is rapid, particularly since it is going through damaged tissue and is causing increased peristalsis by its irritating action. Following absorption, the mercury is distributed relatively equally throughout the body tissues, ordinarily in such small concentrations that it causes no appreciable damage. Excretion begins almost immediately by three major pathways. The first of these is the renal route. The mercury is collected in the kidneys and passes out in the urine. Concentrations of mercury reach a level sufficient to be toxic so that cellular damage (tubular necrosis) results. In like manner, cellular damage (gangrenous colitis) may occur in the colon, which is an important if somewhat later excretory avenue. The third and least important excretory area is the mouth where damage (stomatitis) frequently occurs but is usually not serious. Hence there are three regions in which mercury reaches toxic concentrations and from which it should be removed as rapidly as possible in order to minimize cellular destruction and also to preserve organic integrity.

Of these regions the kidneys are the least accessible and the earliest involved. Direct lavage is technically difficult even in so far as the pelves and calices, where damage is minimal, are concerned, and, at present, technically impossible with regard to the tubules where damage is maximal. Physiologic lavage by massive diuresis is then the method of choice. Fluids in large amounts by mouth and parenterally serve as the best diuretics. Early use of fluids is imperative because the action of the mercury is such as to decrease the excretory powers of the kidneys to the vanishing point (anuria).

The colon is affected by the mercury at first directly by contact and later indirectly by excretion. Early diarrhea is a prominent symptom of acute mercury poisoning and is evidence of the presence of mercury which has passed through from the stomach to the colon. Later, excretion of mercury begins with continuation or renewal of the symptoms of colitis. Here again, the method of choice for treatment is lavage or irrigation in an attempt to wash out the mercury. High colonic irrigations begun immediately and repeated at regular intervals fill this requirement.

Finally, lavage or irrigation of the mouth for both direct and indirect toxic effects of the mercury is indicated in order to remove the metal from this area as rapidly as possible.

The foregoing outline is a very general one of a rational method of treatment of acute mercury poisoning. It is obvious that a neutralizing or antidotal agent for mercury to be used in conjunction with the general method would be of great value. Therefore, after careful consideration of these things, Rosenthal<sup>1</sup> in 1933 reported the results of the use of various antidotes in experimentally produced mercury poisoning in dogs and rats. He advocated the use of sodium formaldehyde sulfoxylate because of its relative stability, low toxicity, and rapid reaction with mercury in vivo and in vitro. He reported the effects of this drug in one human case and outlined a routine of therapy to be followed with the drug. A little later<sup>2</sup> he reported excellent results in 10 human cases following essentially the same routine which he had suggested previously.

This consisted of:

1. Gastric lavage with 5 per cent sodium formaldehyde sulfoxylate, leaving 200 c.c. of solution in the stomach.

2. Ten grams of sodium formaldehyde sulfoxylate in 100 to 200 c.c. of water intravenously in twenty to thirty minutes. If poisoning is severe, this is repeated in four to six hours with 5 to 10 Gm.

3. If colitis develops, high colonic irrigations are to be given once or twice a day with 1:1,000 sodium formaldehyde sulfoxylate.

Brown and Kolmer<sup>3</sup> repeated some of the experimental work on rabbits, and they concluded that sodium formaldehyde sulfoxylate had a definite toxicity which was increased in the presence of mercuric chloride. They felt that there was a certain danger in using the drug intravenously, and the best effects were obtained by oral use as soon as possible. Monte and Hull<sup>4</sup> treated seven cases with the method of Rosenthal, utilizing also physiologic supportive measures. Four of the seven patients showed evidences of severe poisoning, three of them dying. Soon afterward, Rosenthal<sup>5</sup> confirmed the results of Brown and Kolmer, showing that the toxicity of sodium formaldehyde sulfoxylate was increased in the presence of mercuric chloride, but he stated that the increase was not as great as they reported. He suggested that the second intravenous dose should contain 5 Gm. or less of the drug and he showed that the speed of injection affected the toxicity directly. In addition, he cited 25 human cases of his own and about 25 from other clinics in which the results of treatment were good without untoward effects. Following this, in 1937, Modell and associates<sup>6</sup> performed further experiments on animals which had been given fatal doses of mercury. They concluded that the protection afforded by sodium formaldehyde sulfoxylate was limited by the speed of action of the mercury. Moreover, they showed that the end product of the reaction between mercury and sodium formaldehyde sulfoxylate was one-half to one-third as toxic as the mercury itself. Of most importance, however, was their demonstration that the alkalization of the media increased the speed of reaction of the sodium formaldehyde sulfoxylate with mercury. The last point is of definite significance, especially in cases of oral ingestion of mercury where the poison enters the strongly acid stomach secretion.

Hug<sup>7</sup> reported a series of 72 cases of acute mercury poisoning treated with sodium formaldehyde sulfoxylate by gastric lavage and intravenous injection. There were five deaths, although about one-half the patients showed definite evidence of kidney damage by urinary changes. On the other hand, Monte and Hull<sup>8</sup> reported 40 patients treated with sodium formaldehyde sulfoxylate by lavage and intravenous injection as well as by physiologic supportive measures, such as intravenous glucose and saline solutions, morphine, and transfusions. Eighteen patients developed symptoms of acute poisoning, and of these, 14 died. They concluded that there may be a definite danger in the use of the drug because of the apparent increase in mortality over other averages<sup>9</sup> and also that the drug is valuable only if used very early. Other authors<sup>10-14</sup> have reported cases treated with sodium formaldehyde sulfoxylate by lavage, by intravenous injection, and by colonic irrigation with generally favorable results.

Sodium formaldehyde sulfoxylate is a stable, white, crystalline powder, readily soluble in water. It is relatively nontoxic whether used orally, rectally, or intravenously. It reacts directly with mercuric chloride (more readily in alkaline medium) to precipitate mercury or to form a mercury compound which is one-third to one-half as toxic as the original mercury salt. The last fact accounts for the increase of toxicity of the sodium formaldehyde sulfoxylate in the presence of mercuric chloride. The drug is largely excreted in the urine. Due to these properties sodium formaldehyde sulfoxylate is at present one of the best antidotal agents for acute mercury poisoning. It may be used in all lavage and irrigation fluids as well as intravenously to reach the kidneys. It is easy to handle and it is readily available.

In view of these facts, sodium formaldehyde sulfoxylate was tried in a series of 20 patients over a period of about eighteen months. The routine used was based on that of Rosenthal,<sup>1, 2, 5</sup> with a number of modifications based on other experimental work<sup>6</sup> and on certain personal ideas. It consisted of:

1. Immediate gastric lavage, using 4 per cent sodium formaldehyde sulfoxylate in 2 to 3 per cent sodium bicarbonate solution. Five hundred cubic centimeters or more of solution are used, leaving 250 c.c. in the stomach.

2. Injection of 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate intravenously, taking one-half hour for the injection and then following it with a continuous drip of 5 per cent glucose in normal saline. Injection of an additional 100 c.c. of 5 per cent sodium formaldehyde sulfoxylate intravenously six hours later.

3. Use of two high colonic irrigations a day, using 4 to 5 liters of 1:1,000 (0.1 per cent) sodium formaldehyde sulfoxylate for each irrigation.

4. Gastric lavage with 500 c.c. of 4 per cent sodium formaldehyde sulfoxylate in 2 to 3 per cent sodium bicarbonate solution twice a day for two days.

5. Injection of at least 2,000 c.c. of 5 per cent glucose in normal saline intravenously daily for seven to ten days.

6. In the event of stomatitis, use of a mouth wash of 2 per cent sodium formaldehyde sulfoxylate in 2 to 3 per cent sodium bicarbonate solution two to three times daily.

7. Use of morphine, stimulants, and other supportive measures as needed. If shock is present, it is combatted by appropriate methods.

8. Use of soft diet and forced fluids.

A number of laboratory procedures are performed. Daily routine urine examination is done, and daily nonprotein nitrogen, carbon dioxide combining power, and creatinine determinations are made on the blood. Specimens of urine, stool, vomitus, colonic washings, stomach washings, and mouth washings are examined for mercury daily using the electrolytic method. Naturally, the procedure is modified to fit the needs of the individual patient and in accordance with the clinical course.

All the patients had taken mercuric chloride in doses ranging from 7.5 (one tablet) to 45 (six tablets) grains. The average dose was 17.68 grains and

the mean dose was 15 grains. Emesis occurred in all patients at intervals varying from five minutes to two hours after ingestion. In 13 patients emesis occurred within fifteen minutes. The interval between ingestion and therapy with sodium formaldehyde sulfoxylate ranged between fifteen minutes and thirty-six hours. Sixteen patients were treated within one hour, and 11 within one-half hour (see Table I).

TABLE I

| CASE | DOSE<br>IN<br>GRAINS | EMESIS<br>INTERVAL | INTERVAL<br>BEFORE<br>THERAPY | URINE                                  | N.P.N.<br>IN MG.<br>PER<br>100 C.C. | CREAT-<br>ININE<br>IN MG.<br>PER<br>100 C.C. | COLITIS | MERCURY<br>PRESENT IN    |
|------|----------------------|--------------------|-------------------------------|--|-------------------------------------|--|---------|--------------------------|
| 1    | 7.5                  | 10 min.            | 8 hours                       | Negative                               | 25-30                               | 1.5-1.6                                      | --      | Stool                    |
| 2    | 15                   | 2 hours            | 6 hours                       | 1-4+ albumin<br>casts, W.B.C.          | 25-46                               | 1.6-2.3                                      | Present | Stool, vomitus,<br>urine |
| 3    | 15                   | 30 min.            | 45 min.                       | Trace albumin,<br>few W.B.C.           | Not<br>done                         | Not<br>done                                  | --      | Vomit                    |
| 4    | 45                   | 10 min.            | 30 min.                       | Trace albumin,<br>W.B.C.               | 32                                  | 1.6  | --      | Negative                 |
| 5    | 15                   | 15 min.            | 45 min.                       | 1-3+ albumin,<br>R.B.C., and<br>W.B.C. | 23-32                               | 1.5-1.6                                      | Present | Stool, vomitus,<br>urine |
| 6    | 15                   | 10 min.            | 30 min.                       | Negative                               | 24-35                               | 1.5  | --      | Negative                 |
| 7    | 22.5                 | 10 min.            | 20 min.                       | 1-2+ albumin,<br>granular casts        | 29-44                               | 1.5-1.7                                      | Present | Stool                    |
| 8    | 15                   | 5 min.             | 45 min.                       | Trace albumin                          | 24-35                               | 1.5  | Present | Stool                    |
| 9    | 22.5                 | 15 min.            | 1 hour                        | 1-3+ albumin,<br>R.B.C., and<br>W.B.C. | 36-67                               | 1.7-8.3                                      | Present | Stool, vomitus,<br>urine |
| 10   | 7.5                  | 10 min.            | 30 min.                       | Trace albumin                          | 18-26                               | 1.4-1.6                                      | Present | Stool, vomitus,<br>urine |
| 11   | 30                   | 20 min.            | 30 min.                       | 3+ albumin,<br>W.B.C., casts           | 28-93                               | 2.8-13.6                                     | Present | Stool, vomitus,<br>urine |
| 12   | 45                   | 30-60<br>min.      | 3 hours                       | Not done                               | Not<br>done                         | Not<br>done                                  | Present | Not examined             |
| 13   | 7.5                  | 15 min.            | 15 min.                       | Negative                               | 21-24                               | 1.5  | Present | Vomit, urine             |
| 14   | 7.5                  | 20 min.            | 40 min.                       | Trace albumin                          | 19                                  | 1.5  | --      | Negative                 |
| 15   | 7.5                  | 30 min.            | 30 min.                       | 1+ albumin                             | 28-31                               | 1.5  | --      | Stool, vomitus,<br>urine |
| 16   | 7.5                  | 10 min.            | 36 hours                      | Negative                               | 28-37                               | 1.5  | Present | Stool, urine             |
| 17   | 15                   | 30 min.            | 30 min.                       | 1+ albumin,<br>W.B.C.                  | 23-34                               | 1.5-1.6                                      | Present | Stool, vomitus,<br>urine |
| 18   | 7.5                  | 5-10<br>min.       | 15 min.                       | Negative                               | 30-35                               | 1.5-1.6                                      | --      | Stool, vomitus,<br>urine |
| 19   | 30                   | 10 min.            | 30 min.                       | 3+ albumin,<br>W.B.C., casts           | 57-121                              | 2.0-7.2                                      | Present | Stool, vomitus,<br>urine |
| 20   | 15                   | 15 min.            | 30 min.                       | Trace albumin,<br>hyaline casts        | 28-34                               | 1.5  | Present | Stool, vomitus,<br>urine |

Of the 20 patients 14 (70 per cent) showed evidence of renal damage by changes in the urine, ranging from traces of albumin to 4+ albumin, casts, red and white blood cells. (No urine test was done in one patient who died.) Four cases (20 per cent) showed evidences of renal damage by definite changes in the blood chemistry. No urine examination was done in one patient and no blood chemistry test in two. Thirteen patients (65 per cent) developed colitis, as evidenced by generalized abdominal cramps, and in some cases, bloody diarrhea. Mercury was found in the vomitus, washings, or excreta in 16 of 19 patients examined (84 per cent). In 12 patients (75 per cent of the positive cases) mercury was present in two or more of these groups (see Table I).

Sixteen patients recovered completely with no residual effects. Four patients died. Of these, three developed anuria and uremia, and the other died shortly after ingestion of the mercury in respiratory failure, evidently the result of aspiration of vomited material or edema of the glottis. The case reports of the patients who died are presented here.

CASE 9.—M. W., a 27-year-old white man, swallowed 3 tablets (22.5 grains) of mercuric chloride with a glass of water in an attempted suicide. Fifteen minutes later he vomited blue material, and about one hour later he was brought into the hospital. On admission he appeared acutely ill and he was vomiting bloody mucoid material. His face was blotchy, and his lips, tongue, and pharynx were markedly injected and moderately swollen. Abdomen was tender. He was lavaged with 1,250 c.c. of 4 per cent sodium formaldehyde sulfoxylate in 2 to 3 per cent sodium bicarbonate solution, and he swallowed about 450 c.c. of the same solution. Some of the lavage returns were gray with precipitated mercury. He was then given 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate intravenously and 100 c.c. of the same solution seven hours later. He was lavaged twice a day for one day, with 4 per cent sodium formaldehyde sulfoxylate in sodium bicarbonate solution, and used a mouthwash and gargle of 2 per cent sodium formaldehyde sulfoxylate in sodium bicarbonate solution two to three times a day. In addition, he was given high colonic irrigations with 1:1,000 sodium formaldehyde sulfoxylate twice a day for three days. In spite of all treatment, including 5 per cent glucose in normal saline intravenously daily, the patient became steadily worse, developed marked oliguria on the third hospital day and slight edema with a moderate temperature elevation on the fourth hospital day. On the morning of the fifth hospital day he developed acute pulmonary edema, even though the intravenous fluid had been changed to 5 per cent glucose in distilled water. He responded well to treatment for this complication, but a short time later he gasped suddenly and died. Permission for autopsy was not obtained. Urines when obtained showed 1-3+ albumin and a few red blood cells and white blood cells. Nonprotein nitrogen started at 36 mg. per 100 c.c. and rose to 67 mg. per 100 c.c. Creatinine started at 1.7 mg. per 100 c.c. and at time of death was 8.3 mg. per 100 c.c. Mercury was found in the vomitus, stool, lavage material, and urine.

CASE 11.—H. S., a 28-year-old white woman, took four tablets (30 grains) of mercuric chloride with suicidal intent. She vomited twenty minutes later and was brought into the hospital about one-half hour later. On admission examination revealed an obese woman, appearing acutely ill, vomiting continuously. Her lips were swollen and she showed marked injection of her mouth and throat. Abdomen was slightly tender and showed slight spasm. Remainder of examination was negative. Patient was immediately lavaged with 500 c.c. of 4 per cent sodium formaldehyde sulfoxylate in 4 per cent sodium bicarbonate solution. The washings were dark gray with precipitated mercury. She was washed further with large amounts of normal saline and sodium bicarbonate solution. A continuous intravenous drip of 5 per cent glucose in normal saline was started. Three hours later she was given 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate intravenously, followed by 100 c.c. six hours later. Because of the character of the washings (containing precipitated mercury), a tube was kept in the stomach for thirty-six hours, and the stomach was washed alternately with plain normal saline and sodium bicarbonate solution and 4 per cent sodium formaldehyde sulfoxylate in sodium bicarbonate solution every three hours. She was given two high colonic irrigations a day with 1:1,000 sodium formaldehyde sulfoxylate and continuous intravenous 5 per cent glucose in normal saline, followed later with 5 per cent glucose in distilled water. She was given mouthwashes of 2 per cent sodium formaldehyde sulfoxylate because of the stomatitis and pharyngitis. On the second hospital day she had an oliguria, and on the third day she became completely anuric. She became progressively worse with onset of drowsiness, edema, and abdominal cramps. She developed hemorrhages in the fundi. Despite diathermy to the kidneys and supportive measures, including sodium lactate intravenously to combat the acidosis, the patient died on the eighth hospital day. Her urine when obtained initially showed 3+ albumin, 4 to 6 white blood cells and occasional casts. Her



nonprotein nitrogen ranged from 28 mg. per 100 c.c. on admission to 93 mg. per 100 c.c. at the time of death; her creatinine from 2.8 mg. per 100 c.c. to 13.6 mg. per 100 c.c. Carbon dioxide combining power decreased to 25.1 volumes per cent, but returned to 41.9 volumes per cent at the time of death. Mercury was found in the vomitus, gastric washings, colonic washings, stools, and urine. Permission for autopsy was obtained. The essential findings at autopsy were:

*Gross:* Pulmonary edema and patchy bronchopneumonia; large, pale, swollen kidneys, with moderately injected pelves, weighing together 100 Gm.; moderate gastritis, enteritis, and colitis with a greenish gray, pseudodiphtheritic membrane in the rectum and a portion of the sigmoid colon.

*Microscopic:* Lungs showed pulmonary edema and bronchopneumonia; kidneys showed a marked mercury nephrosis with early regeneration and healing; stomach, small intestine, and colon showed simple inflammation, with slight superficial necrosis of the mucosa in the region of the rectum and sigmoid colon.

CASE 12.—H. B., a 37-year-old white male, took 6 tablets (45 grains) of mercuric chloride with suicidal intent. He vomited thirty to sixty minutes later and was brought into the hospital about three hours after ingestion. Examination on admission revealed a comatose man, breathing heavily and with some difficulty. He was cyanotic and vomited bloody material, and was having bloody diarrhea. Skin was moist and cold. There were coarse râles at both lung bases. Blood pressure was 140/100. He was immediately lavaged with large amounts of 4 per cent sodium formaldehyde sulfoxylate and sodium hyposulfite. He was given 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate intravenously, followed by 5 per cent glucose in normal saline in a continuous drip. Preparations were made for transfusion. However, the patient became rapidly and progressively worse and died one hour and forty minutes after admission. Death was evidently due to respiratory failure following either aspiration of vomitus or acute edema of the glottis. It was later discovered that the patient had a history of peptic ulcer. No laboratory studies were done. Autopsy was not performed.

CASE 19.—A. M., a 29-year-old white female, took four tablets (30 grains) of mercuric chloride with suicidal intent. She vomited blue material ten minutes later and was brought into the hospital about thirty minutes after ingestion. On admission examination revealed an acutely ill woman, vomiting large amounts of green fluid and having severe diarrhea. Mouth and pharynx showed considerable injection and swelling. Remainder of the examination was essentially negative. The patient was immediately lavaged with 500 c.c. of 4 per cent sodium formaldehyde sulfoxylate in 2 to 3 per cent sodium bicarbonate solution. About 100 c.c. of solution were left in the stomach. She was then given 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate solution intravenously, followed by a continuous drip of 5 per cent glucose in normal saline. Six hours later an additional 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate were given intravenously. A Levine tube was put down into the stomach and the stomach was washed with 4 per cent sodium formaldehyde sulfoxylate in sodium bicarbonate solution every two hours four times. About twenty-four hours after admission the patient was again given 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate solution intravenously and about 500 c.c. of Fischer's solution as well. She was lavaged with 4 per cent sodium formaldehyde sulfoxylate in sodium bicarbonate twice on the day after admission. She was given two high colonic irrigations a day with 1:1,000 sodium formaldehyde sulfoxylate. She was put on a liquid diet with forced fluids and was given large amounts of 5 per cent glucose in normal saline, alternating with 5 per cent glucose in distilled water up to 4,000 c.c. a day. About eighteen hours after admission the patient began to show definite oliguria, and on the fourth hospital day she became anuric. She became progressively worse, appeared toxic and drowsy, and developed a generalized edema on the sixth hospital day and, despite all supportive measures, died on the seventh hospital day. Urines showed 3+ albumin and many granular casts as well as many white blood cells. Nonprotein nitrogen ranged from 57 mg. per 100 c.c. to 121 mg. per 100 c.c., creatinine

from 2.0 mg. per 100 c.c. to 7.2 mg. per 100 c.c.. Vomitus, gastric washings, urine, stools, and colonic washings were positive for mercury. Permission for autopsy was obtained. The essential findings at autopsy were:

*Gross:* Generalized edema; moderate pulmonary edema and patchy bronchopneumonia; large, pale, swollen kidneys; a deep ulceration in the stomach, about 1 cm. in diameter, with injection of the stomach mucosa elsewhere and moderate injection of the colon.

*Microscopic:* Lungs showed edema and bronchopneumonia; kidneys showed a marked mercury nephrosis with early regenerative changes; the stomach showed a deep necrotic area, extending into the muscularis, with a mild inflammatory change elsewhere in the stomach mucosa, and the colon showed a mild catarrhal colitis.

#### COMMENT

The important element in any method of treatment of acute oral mercury poisoning is not the rapidity with which patients recover nor the presence or absence of residual effects, but it is the actual recovery itself or the resulting mortality rate. The mortality rate of 20 per cent obtained in this small series is slightly better than the 25 per cent rate obtained by Hull and Monte<sup>9</sup> in a much larger series treated by various other methods. In the same large series they also noted that about 50 per cent of all cases showed evidence, either urinary or otherwise, of real poisoning, half of these dying. In this series 17 cases, or 85 per cent, showed evidences of poisoning with either renal or colonic damage and 10 cases, or 50 per cent, showed both. Nevertheless, in a small series these figures are not too significant.

In the four fatal cases the doses were larger than the average of 17.68 grains, ranging from 22.5 to 45 grains, the time interval from ingestion to emesis was ten minutes or over, and the treatment interval ranged from thirty minutes to three hours. Moreover, in Case 11 intravenous therapy was delayed for over three hours; hence the absorbed mercury was able to reach the kidneys and cause damage before it could be precipitated. In Case 12 death was primarily respiratory. The importance and value of high colonic irrigations are amply demonstrated by the autopsy findings in Cases 11 and 19 where the colons were only superficially involved, indicating that the irrigations were effective in removing the concentrated mercury. The high incidence of colitis even early in the course of the poisoning makes the prompt institution of high colonic irrigations especially significant. Mercury was found in the stools of 14 of the 16 mercury positive cases, and of the mercury positive cases which showed clinical evidence of colitis it was found in the stools of all except one (11 out of 12). This again emphasizes the role of the colon in the excretion of the mercury.

The importance of early and intensive treatment both oral and intravenous cannot be overestimated, inasmuch as the mercury is absorbed rapidly and produces severe damage in a short time. The fact that the substance formed by combination of mercury and sodium formaldehyde sulfoxylate is still toxic should not be overlooked, so that physiologic supportive measures must be maintained encouraging excretion by all avenues. Moreover, the toxic effect of the mercury on the body as a whole must be combatted by the supportive measures.

The method of treatment advocated here is far from ideal. It is attended by certain dangers and requires careful supervision. Each case should be treated individually and each situation should be met as it arises without too

much dependence being placed on the antidotal drug. However, under careful supervision, the general method has a definite value and the drug deserves further trial until a more specific agent is discovered.

#### SUMMARY

A method of treatment for acute mercury poisoning utilizing sodium formaldehyde sulfoxylate and based on the work of Rosenthal is outlined. It consists of: 1. Immediate gastric lavage with sodium formaldehyde sulfoxylate. 2. Intravenous injection of sodium formaldehyde sulfoxylate. 3. High colonic irrigations with the same drug twice daily. 4. Gastric lavage twice daily for two days. 5. Daily intravenous glucose in saline. 6. Mouthwash with sodium formaldehyde sulfoxylate for stomatitis. 7. Supportive measures as needed. 8. Fluids and soft diet.

The findings and results in 20 cases treated by this method are presented.

Four fatal cases are presented and the causes of death are analyzed.

The significance of early treatment, physiologic supportive measures, high colonic irrigations, and individual management are stressed.

The method is not considered to be ideal, but it is felt that it should be used in all cases of acute mercury poisoning until a more efficient routine is discovered.

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## INFLUENCE OF COMPONENTS OF THE VITAMIN B COMPLEX ON RECOVERY FROM FATIGUE\*

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IT IS generally conceded that one of the cardinal symptoms of thiamine deficiency is easy fatigability.<sup>1-4</sup> It is reported that in patients suffering from thiamine deficiency the blood pyruvate is elevated and that the administration of thiamine causes it to become lower.<sup>5, 6</sup> Muscular exercise produces a rise in the blood pyruvate level above that found at rest.<sup>7</sup> Further, thiamine-deficient patients develop a higher level of pyruvic acid in the blood after exercise than do normal persons<sup>8</sup> and are capable of less work.<sup>9</sup> Some investigators<sup>10-12</sup> have felt that the administration of thiamine and other components of the vitamin B complex allayed fatigue. For these reasons, an attempt was made to ascertain whether *recovery* could be hastened by the administration of thiamine and other members of the vitamin B complex to fatigued persons.

Five persons under controlled conditions of housing and diet were studied. The diet was adequate in all respects and contained approximately 1.8 mg. of thiamine and 3.5 mg. of riboflavin. "Double work periods"<sup>13</sup> were used. The work was done on Monday, Wednesday, and Friday afternoons at the same time between 2 and 4 o'clock. The subjects would work to fatigue at a rate of 1,235 kg. per minute, with a pedaling rate of 54 r.p.m. on the bicycle ergometer, described by Kelso and Hellebrandt.<sup>14</sup> The end point was their inability to maintain this rate. Immediately after this first work period the vitamin to be tested was injected intravenously. Then ten minutes after the cessation of the first work period each subject would again work to fatigue. Placebo injections were substituted at random for the vitamin injections. No subject was allowed to watch an injection. The vitamins used were thiamine hydrochloride,† 25 mg., 20 trials; B complex parenteral,† 1 to 5 c.c., 7 trials; cocarboxylase,‡ 10 mg., 4 trials; riboflavin,† 1.25 to 10 mg., 12 trials. The results are shown in Tables I and II.

The results for thiamine, cocarboxylase, and B complex are included in Table I. Since they had no significant effect, the results with each vitamin are

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Thiamine hydrochloride—50 mg. per cubic centimeter. Riboflavin—1 mg. per cubic centimeter. B complex parenteral, each 10 c.c. contains:

Thiamine hydrochloride, 30 mg.

Nicotinamide, 100 mg.

Riboflavin, 3.3 mg.

Pyridoxine hydrochloride, 10 mg.

‡Supplied by Merck and Company.

not tabulated. The riboflavin was administered at a later period of training, and the data on it are in Table II. Referring to Table I, it will be noted that in 2 of the 3 subjects (B and D) the work output of the second work period following the administration of vitamins is insignificantly less than with the placebo; in the other subject (A) it is not significantly elevated. In no case is the total work output greater following vitamin administration than it is in the control or placebo series. These two factors more than cancel the apparently favorable effect of vitamins on per cent recovery, which effect, however, is not statistically significant.

TABLE I  
DATA ON THIAMINE, COCARBOXYLASE, AND THE B COMPLEX

| SUBJECT | TYPE OF WORK PERIOD | PERIOD OF TRAINING (MONTHS) | NUMBER OF DOUBLE WORK PERIODS | AVERAGES                     |                              |                         |                   |
|---------|---------------------|-----------------------------|-------------------------------|------------------------------|------------------------------|-------------------------|-------------------|
|         |                     |                             |                               | 1ST PERIOD WORK OUTPUT (KG.) | 2ND PERIOD WORK OUTPUT (KG.) | TOTAL WORK OUTPUT (KG.) | PER CENT RECOVERY |
| A       | Control Vitamin     | 2 -3                        | 19                            | 13,951                       | 6,356                        | 20,275                  | 46                |
|         |                     | 3 -3.5                      | 9                             | 13,033                       | 7,156                        | 20,168                  | 56                |
| B       | Control Vitamin     | 1.5 -3.75                   | 21                            | 6,573                        | 4,491                        | 11,142                  | 68                |
|         |                     | 1.5 -4                      | 15                            | 6,501                        | 4,373                        | 10,889                  | 68                |
| D       | Control Vitamin     | 2 -3.25                     | 15                            | 10,029                       | 5,595                        | 15,540                  | 56                |
|         |                     | 3.25-3.75                   | 7                             | 8,816                        | 5,530                        | 14,246                  | 63                |

TABLE II  
DATA ON RIBOFLAVIN

| SUBJECT | TYPE OF WORK PERIOD | PERIOD OF TRAINING (MONTHS) | NUMBER OF DOUBLE WORK PERIODS | AVERAGES                     |                              |                         |                   |
|---------|---------------------|-----------------------------|-------------------------------|------------------------------|------------------------------|-------------------------|-------------------|
|         |                     |                             |                               | 1ST PERIOD WORK OUTPUT (KG.) | 2ND PERIOD WORK OUTPUT (KG.) | TOTAL WORK OUTPUT (KG.) | PER CENT RECOVERY |
| A       | Control Riboflavin  | 8 -8.5                      | 7                             | 8,788                        | 5,900                        | 14,655                  | 70                |
|         |                     | 8.5 -8.75                   | 3                             | 8,355                        | 5,941                        | 14,297                  | 71                |
| B       | Control Riboflavin  | 8.25-8.75                   | 7                             | 6,079                        | 4,260                        | 10,339                  | 71                |
|         |                     | 8.75-9                      | 3                             | 6,283                        | 4,384                        | 10,667                  | 70                |
| E       | Control Riboflavin  | 4.5 -5                      | 6                             | 3,770                        | 2,669                        | 6,437                   | 71                |
|         |                     | 5 -5.25                     | 3                             | 3,931                        | 2,805                        | 6,736                   | 71                |
| F       | Control Riboflavin  | 4.5 -5                      | 6                             | 4,840                        | 2,696                        | 7,536                   | 56                |
|         |                     | 5 -5.25                     | 3                             | 5,254                        | 2,973                        | 8,032                   | 53                |

Referring to Table II, it is seen that in the case of A the output of work was insignificantly less following riboflavin than in the control series. Considering the subjects B, E, and F, it is apparent that insignificant increases in work output occurred during the first and second periods and in the total work output after riboflavin. However, the insignificant increment observed after the riboflavin can well be explained by training or experimental variation. Also, if an effect had been produced by the riboflavin, a significant increase in the per cent recovery should have occurred. Such was not the case. Thus no significant effects were observed, and the subjects reported no subjective sensations whatever after receiving any of the vitamins.

These results show that the vitamins administered above do not act promptly to facilitate recovery. This statement is made because it is not to be concluded

that an increase in recovery will not occur when these vitamins are added to a diet containing an insufficient quantity of them.

*Further Observations on the Double Work Period Method of Studying Fatigue.*—One reason for using the double work period method of studying fatigue is that it assists the investigator in determining whether or not his subjects have worked to complete fatigue. This is based in part on our observations<sup>13</sup> that the per cent recovery is more constant than the output of a single work period. For example, if a subject does not work to complete fatigue in the first period, he will work longer than usual in the second period, and his per cent recovery will be correspondingly high. If, however, he does work to complete fatigue the first period and does not work to complete fatigue in the second period, his per cent recovery will be unusually low. To check whether or not per cent recovery could be "faked," four subjects were stopped when they had done approximately 75 per cent as much work as average in the first period. In the second period they were asked to stop as far short of fatigue as they had in the first period. In no case was their usual per cent recovery approximated.

TABLE III  
INITIAL DOUBLE WORK PERIODS  
(25 Subjects)

|                        | RANGE           | COEFFICIENT OF VARIATION (%) |
|------------------------|-----------------|------------------------------|
| 1st period work output | 1,112-6,133 kg. | 40.9                         |
| 2nd period work output | 761-2,511 kg.   | 23.0                         |
| Total work output      | 1,873-8,023 kg. | 31.1                         |
| Per cent recovery      | 31-99 per cent  | 19.0                         |

To test the double work period method further, the data of the initial double work periods on 25 new subjects at the above standard rate of work were analyzed. The variability of the four items of fatigue data obtained by this method are shown in Table III. These data indicate that even in untrained persons the per cent recovery is the most constant physiologic characteristic of these four manifestations of fatigue and that the first work period, the variability of which corresponds with that of the single work period, is the most variable. That the ten-minute rest period is not too short to reveal the effect of any substance on recovery has been demonstrated by the favorable effects of caffeine.<sup>13</sup>

#### CONCLUSIONS

Thiamine, cocarboxylase, riboflavin, and parenteral vitamin B complex given intravenously have no immediate influence on recovery from voluntary muscular fatigue in subjects receiving "adequate" nutrition.

The double work period method of studying fatigue enables the investigator to determine whether his subjects are actually working to a designated end point of fatigue.

The per cent recovery is less variable than the first period work output, the second period work output, or the total work output in initially untrained subjects.

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# CLINICAL CHEMISTRY

## CIRRHOSIS OF THE LIVER\*

### RESULTS OF TREATMENT WITH PARENTERALLY ADMINISTERED AMINO ACIDS

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#### PLASMA PROTEINS AND CIRRHOSIS OF THE LIVER

RECENT studies in the relationship between plasma proteins and liver diseases have indicated that in order to maintain an efficient liver function, adequate protein supplies are necessary; and conversely, in order to maintain normal plasma protein levels, the liver must be in a good functional condition.<sup>6</sup>

The finding of decreased concentrations of blood proteins in cirrhosis of the liver was first observed by Grenet and Gilbert in 1907, and has been frequently substantiated since.<sup>1, 2, 8, 10</sup> In general, total protein content of the serum is usually decreased, primarily because of a depletion of the albumin content, and the A./G. ratio is low or inverted. Occasionally, the serum globulin is increased, apparently compensating for the albumin deficiency, so that total serum proteins may be within normal limits.<sup>8, 10</sup> The hypoalbuminemia or hypoproteinemia generally found in hepatic cirrhosis may be attributable to one, or to a combination of the following factors:<sup>11</sup> (a) Deficiency of protein food intake. (b) Protein loss via drainage of albumin in the ascitic fluid. (c) Increased permeability of the capillary bed with consequent diffusion of proteins into the tissues. (d) Failure of the liver to produce proteins or defective formation of proteins. (e) Deficiency of protein-building materials stored in the normal liver. (f) Other factors as yet undefined.

Deficiency of protein intake has been excluded as a significant factor by Foley and co-workers,<sup>10</sup> who described several patients with severe dietary restrictions, but without clinically recognizable hepatic damage, who, although they exhibited some decrease in total proteins, showed no increase in the globulin concentration and no reversal of the A./G. ratio. Myers and Keefer<sup>1</sup> were unable to demonstrate any effect on plasma proteins by feeding patients with cirrhosis of the liver diets containing from 180 to 300 Gm. of proteins daily, possibly because of the impairment of the proteogenic function of the liver.<sup>2, 10</sup> The significance of protein loss via ascitic fluid drainage has been minimized by Barrett, Jones, and Cohn<sup>3</sup> who reported a patient with hepatic cirrhosis who

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required frequent paracentesis with removal of an average of 10.2 Gm. of protein per day in the ascitic fluid over a 204-day period, without any definite fall in the plasma protein level. Patients without cirrhosis, but with ascites due to carcinomatous peritoneal or hepatic metastases, or tuberculous peritonitis, or congestive heart failure do not exhibit diminution of plasma protein content, although the protein loss is comparable.<sup>10</sup>

Butt and associates<sup>11</sup> suggested that the usual protein determination does not adequately reflect the osmotic interplay at the capillary membrane. They actually measured the colloidal osmotic pressure of the blood serum in patients with cirrhosis of the liver and found that in almost all cases the serum colloid osmotic pressure was below normal levels. All the patients with edema and ascites had colloid osmotic pressures below the lowest level of normal, probably because of the hypoalbuminemia, since the smaller albumin molecules have the greater osmotic effect. Using the bromsulfalein excretion and hippuric acid synthesis tests to measure the degree of hepatic damage, Butt and his group found an apparent correlation between the colloid osmotic pressure values and the degree of hepatic insufficiency.

The most likely factor, then, to explain hypoproteinemia in hepatic disease is the impairment of hepatic proteogenic mechanism (possibly due to depletion of protein-building stores in the liver), perhaps acting together with increased permeability of the capillary bed.

Pitting edema of the lower extremities is often seen in cirrhosis of the liver, and the most widely accepted explanation has been that the edema is due either to an increased venous pressure in the lower extremities as a result of increased intra-abdominal tension caused by ascites, or to a concomitant congestive heart failure. Myers and Keefer<sup>1</sup> point out that these factors do not explain the dependent edema sometimes seen in patients with a minimal amount of ascitic fluid and without other evidence of circulatory failure. They studied the blood and ascitic fluid protein values in their patients and concluded that the dependent edema is due, partially at least, to hypoproteinemia and increased venous pressure, the latter occurring only in the presence of massive ascites.

#### RATIONALE

Lillie and associates<sup>7</sup> reported the production of cirrhosis in rats maintained on a diet deficient in protein, and also noted an increase in the severity of the hepatic lesions when 20 per cent alcohol solution was used instead of water in conjunction with the protein-deficient diet. Rats maintained on an adequate protein regimen exhibited no liver changes, even when the 20 per cent alcohol was added. The low sulfur content of the protein-poor diet used suggested a possible relationship between the amino acid content of the diet and the ability of the liver to resist orally administered poisons.

In a later report Lowry and co-workers<sup>12</sup> presented the result of treatment of experimental dietary cirrhosis of rat livers produced by a low choline, low casein diet with added cystine. They found that the use of choline, or a high casein diet, or both, resulted in hyperplastic regeneration of the liver cells, with the disappearance of degenerative fatty changes. Grossly, the livers of rats thus treated exhibited a considerable decrease in size.

It has been shown by several observers (Elman and Weiner, Shohl, Blackfan and Butler; Altschuler, Hensel and Sahyun, quoted in reference 13) that nitrogen equilibrium may be maintained by amino acid mixtures. Messinger<sup>14</sup> has reported serum protein regeneration following the use of amino acids in nephrosis. In view of these observations it was deemed advisable to investigate the effects of administering an amino acid mixture to patients with hepatic cirrhosis to determine what effects, if any, would be demonstrable on serum proteins, edema, ascitic fluid production, liver size, and liver function.

#### METHOD

Five patients with clinical diagnoses of cirrhosis of the liver were selected at random from the wards; four were in the so-called "decompensated" stage of portal cirrhosis (i.e., they exhibited ascites at some time or another), and one was clinically in the so-called "compensated" stage. The clinical features of these patients are summarized in this report. In all cases jaundice subsided before the institution of the study. In order to obtain a base line, the patients were observed during a period varying from two months to five months, during which time daily weight fluctuations were charted, ascitic fluid output was measured by paracenteses performed as indication arose, blood protein values were determined, and liver function tests were performed. The bromsulfalein excretion and hippuric acid synthesis tests were used.

Other laboratory determinations made included blood nonprotein nitrogen, blood counts, urines (to exclude proteinuria), icteric indices, and sedimentation rates. As an index of impairment of carbohydrate metabolism in hepatic disease, we used the glucose tolerance test (Exton-Rose method)<sup>5</sup> in preference to the levulose and galactose tolerance tests. During the base-line period of observation, the patients were maintained on a diet of carbohydrate 250 Gm., protein 125 Gm., and fat 60 Gm. (The previous diet was the regular hospital diet, which is adequate in all dietary essentials.) After preliminary determinations were completed, the protein was reduced to 80 Gm., being supplemented by 300 c.c. of a 15 per cent solution of amino acids.

Because of the possibility that patients with cirrhosis of the liver might conceivably exhibit disorders of digestion clinically unrecognizable, the amino acids were administered intravenously in order to be certain that they actually reached the liver. The mixture used was prepared from a casein hydrolysate to which tryptophane was added, and conformed to the following analysis:

|                             |                               |
|-----------------------------|-------------------------------|
| Total nitrogen              | 2.0 per cent                  |
| Alpha amino acid nitrogen   | 83 per cent of total nitrogen |
| Calcium                     | 0.01-0.02 per cent            |
| Tryptophane added           | 1.0 per cent                  |
| Sulfates                    | 0.01 per cent                 |
| Phosphates, iron, magnesium | trace                         |
| Acidity                     | pH 4.0-4.3                    |

The solution was administered daily, intravenously, after the patient's breakfasts, for four weeks (except in the case of patient G. R., in whom the development of an incidental type XIII pneumococcus pneumonia with bacteremia necessitated cessation of the administration of amino acids after three and one-half weeks).

The solution was administered in undiluted form over a period varying from thirty-five minutes to four and one-half hours. Reactions were few and mild. The first dose resulted in an increased number of bowel movements in three of the five patients. The stools were of normal consistency, and the increased number of evacuations was attributed to a hypermotility of the colon. This reaction was observed the first day only and did not recur. Slight nausea, occasional vomiting, or one-half to one degree elevation of temperature, associated with chilly sensations, were occasionally observed during the first week of amino acid administrations. These reactions tended to occur in the patients who were clinically in the poorest condition at the onset of the experiment. The rate of administration of the solution bore no constant relationship to the incidence of such reactions, for a patient occasionally exhibited nausea or a slight fever when the period of administration was four and one-half hours, and no reaction whatsoever when the period was one-half hour. No systemic reactions occurred after the first week of administration of the solution. Local reactions at the sites of intravenous administration of the solution consisted of a slight transient burning sensation if some of the solution leaked into the surrounding tissues, and occasional painless local thrombosis of the veins without associated swelling or inflammatory reaction.

During the month of administration of amino acids frequent determinations of blood proteins were made. The degree of hepatic and splenic enlargement was gauged by measuring the distance of the liver edge or splenic tip from the costal margin in the nipple line. These measurements were made immediately after paracentesis in those patients with ascites to assure greater accuracy.

At the conclusion of the arbitrarily determined month of administration of intravenous amino acids, the bromsulfalein excretion, hippuric acid synthesis, glucose tolerance tests, serum proteins, blood nonprotein nitrogen, icteric index, erythrocyte sedimentation rate, and blood counts were repeated.

#### CASE REPORTS

CASE 1.—G. R., a 53-year-old white male patrolman, admitted on October 21, 1941, complained of an upper respiratory infection of one month's duration; abdominal swelling, anorexia, exertional dyspnea, and ankle edema of one and one-half weeks' duration. History of left apical tuberculosis, treated from 1929 to 1933 with rest, pneumothorax, and phrenicectomy. The tuberculosis was inactive since 1933. The patient admitted only slight previous indulgence in alcohol. Physical examination: icteric sclerae (icteric index 40); ascites grade 3 (on a basis of severity of grades 1 to 4); grade 1 edema of the legs; diminished breath sounds in both bases; dullness in left apex anteriorly with diminished breath sounds; and liver enlarged to 10 cm. below the costal margin. Grade 4 penile and scrotal edema developed during observation period in hospital. Laboratory data: urine negative; blood Kahn test negative; sputum and ascitic fluid negative for tubercle bacilli; blood sugar and nonprotein nitrogen normal; blood count normal; sedimentation rate 22 mm. in an hour. X-ray examination of the chest revealed thickened pleura of the left lateral chest wall and fibrosis at the left apex with angulation of the trachea. Clinical diagnosis: portal cirrhosis, decompensated; pulmonary tuberculosis, left apex, arrested.

CASE 2.—P. W., a 69-year-old white male seaman, admitted September 17, 1941, had jaundice of ten days' duration, anorexia of one month's duration, and intermittent ankle edema for two years. The patient reported moderately excessive alcoholic indulgence previously. Physical examination: senility; generalized peripheral arteriosclerosis; jaundice (icteric index 75); liver enlarged to 3 cm. below the costal margin; splenic tip barely pal-

pable; grade 3 edema of the lower extremities; slight epigastric tenderness; and an enlarged, tender, boggy prostate. Laboratory data: blood Kahn test negative; urine negative, except for bile during period of jaundice; blood sugar and nonprotein nitrogen normal; blood count normal; sedimentation rate 29 mm. in an hour. Electrocardiogram showed some evidences of myocardial damage. The edema of the lower extremities subsided with rest. Clinical diagnosis: generalized arteriosclerosis and arteriosclerotic heart disease; portal cirrhosis, compensated; chronic prostatitis.

CASE 3.—G. B., a 46-year-old white male laborer, admitted on July 23, 1941, had dyspnea, ascites, and ankle edema of one month's duration. He reported excessive use of alcohol in the past. Physical examination: acutely ill, obese, white male, with scleral icterus (icteric index 25); ascites grade 3; grade 2 edema of the lower extremities; umbilical hernia; bilateral foot drop; liver enlarged to 21 cm. below the costal margin. Laboratory data: urine negative; blood Kahn test negative; blood sugar and nonprotein nitrogen normal; sedimentation rate 21 mm. in an hour. The foot drop cleared rapidly with the use of thiamine chloride, and was attributed to a peripheral neuritis, secondary to previous inadequate dietary intake and chronic alcoholism. During his stay in the hospital the patient developed hematuria (probably due to rupture of a urethral varix) which subsided spontaneously within a few days. Clinical diagnosis: portal cirrhosis, decompensated; chronic alcoholism.

CASE 4.—J. R., a 49-year-old white male laborer, admitted September 22, 1941, complained of ascites of three months' duration, jaundice of three weeks' duration, and ankle edema of five days' duration. He reported exceedingly heavy indulgence in alcohol previously. Physical examination: chronically ill white male; jaundiced (icteric index 145); liver enlarged to 12 cm. below the costal margin; spleen enlarged to 7 cm. below the costal margin; grade 4 ascites; caput medusae; grade 3 edema of the lower extremities and abdominal wall; small umbilical hernia; external hemorrhoids; obesity; and dental caries. Laboratory data: urine negative, except for bile during period of jaundice; blood Kahn test negative; blood count indicated slight anemia; blood nonprotein nitrogen and sugar normal; sedimentation rate 18 mm. in an hour. Clinical diagnosis: portal cirrhosis, decompensated; chronic alcoholism.

CASE 5.—A. G., 52-year-old white male bartender, was admitted October 28, 1941, complaining of anorexia, intermittent nausea, and vomiting, weight loss of 30 pounds, ascites, and weakness, all of six months' duration. He reported moderately excessive previous indulgence in alcohol. Physical examination: generalized icterus (icteric index 18); slight diffuse enlargement of the thyroid; liver enlarged to 11 cm. below the costal margin; spleen enlarged to 7 cm. below the costal margin; minimal pretibial edema; small amount of fluid in the left chest; grade 3 ascites; old gunshot wound of the right lower extremity with an ankylosed right knee; flat feet; varicose veins; and left scrotal hernia. Laboratory data: urine negative; blood count normal; blood Kahn test negative; blood sugar and nonprotein nitrogen within normal limits; sedimentation rate 24 mm. in an hour. The ascites did not recur after initial paracentesis. Clinical diagnosis: portal cirrhosis, early decompensation; left scrotal hernia; old gunshot wound, right lower extremity.

## RESULTS

1. *Subjective Effects.*—Clinically, one of the patients (A. G.) reported an excellent improvement in his general condition, associated with moderate gain in strength, great improvement in mental outlook, and an increased feeling of well-being. Three patients (G. R., J. R., and G. B.) reported moderate subjective improvement, and one patient was subjectively unchanged (P. W.). One of the patients (J. R.), however, developed a transitory psychosis three weeks after the cessation of our study, but we believe that this episode represented merely a complicating psychosis in the course of an organic disease rather than any deleterious effect of the therapy per se.

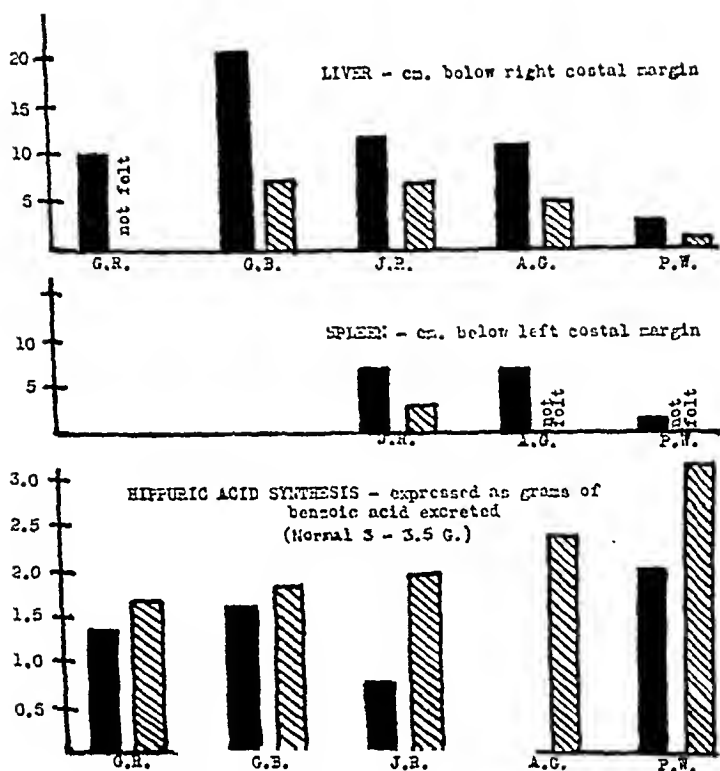


Fig. 1.—The effect of the administration of amino acids on the size of the liver and spleen, and on the hippuric acid synthesis test. Preliminary observations are solid blocks; findings after therapy are represented by lined blocks or the words "not felt."

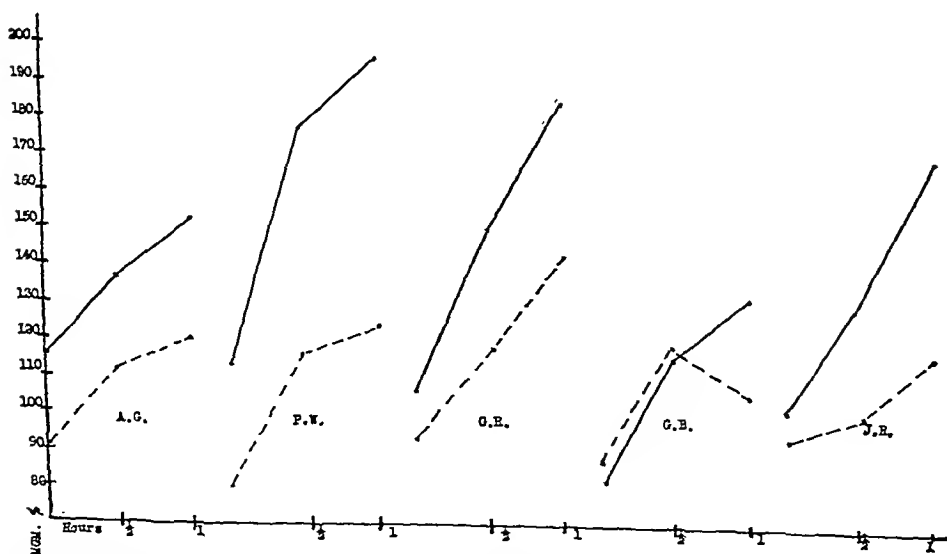


Fig. 2.—The effect of the administration of amino acids on the glucose tolerance curves. Straight lines represent preliminary determinations; broken lines, after therapy.

TABLE I  
EFFECT OF AMINO ACIDS ON ASCITIC FLUID

|   | J. R.                | G. B.                |
|---|----------------------|----------------------|
| Average interval between paracentesis     |                      |                      |
| Observation period                        | 8.5 days             | 7.7 days             |
| Administration period                     | 8.7 days             | 8.7 days             |
| Average daily output of ascitic fluid     |                      |                      |
| Observation period                        | 1,333 c.c.           | 1,451 c.c.           |
| Administration period                     | 1,398 c.c.           | 1,377 c.c.           |
| Average specific gravity of ascitic fluid |                      |                      |
| Observation period                        | 1.015                | 1.011                |
| Administration period                     | 1.011                | 1.012                |
| Average protein content of ascitic fluid  |                      |                      |
| Observation period                        | 730 mg. per 100 c.c. | 775 mg. per 100 c.c. |
| Administration period                     | 600 mg. per 100 c.c. | 610 mg. per 100 c.c. |

2. *Effect on Size of Liver and Spleen.*—The most striking result of the use of the intravenous amino acids was the dramatic decrease in the size of the liver of all the patients, particularly in the four with “decompensated cirrhosis.” No decrease in size of the liver or spleen had been noted during the period of observation preliminary to the use of amino acids, and the decrease in size was much more rapid and uniform than that usually encountered in the natural course of the disease. The hepatic and splenic measurements in centimeters below the costal margin before and after treatment are represented in Table II, and in graphic form in Fig. 1.

TABLE II  
EFFECT OF THE ADMINISTRATION OF AMINO ACIDS ON THE SIZE OF THE LIVER AND SPLEEN, AND ON THE HIPPURIC ACID SYNTHESIS TEST

|   | A. G. | P. W. | G. R. | G. B. | J. R. |
|---|-------|-------|-------|-------|-------|
| Liver—cm. below right costal margin   |       |       |       |       |       |
| Preliminary   | 11    | 3     | 10    | 21    | 12    |
| After therapy   | 5     | 1     | 0     | 7     | 7     |
| Spleen—cm. below left costal margin   |       |       |       |       |       |
| Preliminary   | 7     | 1     | 0     | 0     | 7     |
| After therapy   | 0     | 0     | 0     | 0     | 3     |
| Hippuric acid synthesis—as grams of benzoic acid excreted (normal 3 to 3.5 Gm.) |       |       |       |       |       |
| Preliminary   | 2.20  | 2.00  | 1.36  | 1.60  | 0.75  |
| After therapy   | 2.36  | 3.13  | 1.66  | 1.82  | 1.97  |

In view of the report of Lowry and associates<sup>12</sup> already mentioned, in which the livers of experimentally cirrhotic rats were observed to shrink in size and exhibit disappearance of degenerative fatty changes when treatment with choline or a high-casein diet was instituted, it seems logical to assume that the diminution in size of the livers in these cases is primarily due to resorption of the fat in the liver cells. The decrease in the size of the spleen in those patients who exhibited splenomegaly may be attributable to circulatory readjustments secondary to the decreased size of the liver. The mechanism underlying the resorption of the fat in the liver cells is uncertain, but it probably represents a lipotropic action of the

administered amino acids. Unfortunately, we were unable to follow up our patients with liver biopsies.

The possibility that the shrinkage in the size of the liver reflects a change in the water content of the liver tissue is worthy of consideration, and animal experimentation is necessary to confirm or refute this aspect.

3. *Effect on Peripheral Edema.*—Four patients presented peripheral edema of varying degree at the initiation of the study, and all evidenced complete resorption of the edema fluid within two weeks after the start of amino acid administration. We are inclined to believe that this change reflects improvement in the serum proteins and osmotic balance at the capillary membranes.

4. *Effect on the Ascitic Fluid Output.*—The administration of amino acids had no appreciable effect on the output of ascitic fluid, or on the specific gravity of the ascitic fluid. Only two patients required paracentesis a sufficient number of times to warrant consideration, and the characteristics of the ascitic fluid in these patients is presented in Table I. It will be noted that there is a slight but appreciable decrease in the average protein content of the ascitic fluid after the administration of amino acids. We do not believe that the changes in the amount of fluid, or in the interval between paracentesis, or in the specific gravity of the ascitic fluid before and after therapy are of significance. However, the decrease in average protein content of the ascitic fluid during and after therapy may reflect improvement in serum proteins and capillary permeability.

5. *Laboratory Findings.*—(a) Effect on serum proteins: Serum proteins were determined by the use of photoelectric colorimeter, as described by Looney and Walsh.<sup>2</sup> In our hands the results were too bizarre and variable to warrant consideration. Despite the fact that a large part of the study depended upon our following the serum proteins, we were unable to draw any conclusion thereunto appertaining because of the inaccuracy of the method in our hands. Nevertheless, other findings of sufficient importance developed during the course of the study to warrant presentation of our results.

(b) Effect on hippuric acid synthesis: Reference to Table II indicates that in all cases there was some improvement in the ability of the liver to handle ingested sodium benzoate. In three cases (G. R., J. R., and P. W.) this improvement is of significant degree. These findings are suggestive of a beneficial effect on the detoxification function of the liver, and possibly an improvement in the ability of the liver to manufacture the amino acid glycine, since the hippuric acid test indicates the ability of the liver to conjugate ingested sodium benzoate with glycine, forming hippuric acid, which is excreted in the urine if renal function is adequate.<sup>4</sup>

(c) Effect on glucose tolerance curve: Reference to Fig. 2 indicates that in every instance the glucose tolerance curve changed somewhat in the direction of improved utilization of glucose after the period of amino acid administration. We realize that these tests are subject to criticism in that they represent single examinations and not a summary of daily variations in the glucose tolerance of the individual patient. However, the fact that they all changed in the same direction leads us to believe that intravenous administration of amino acids pro-

moted increased tolerance and utilization of carbohydrates, particularly since the patients were on an adequate diet when the initial determinations were made.

(d) Effect on bromsulfalein excretion: In only one patient (G. R.) was there any appreciable effect on the bromsulfalein excretion. In this patient, prior to the administration of amino acids, there was 40 per cent bromsulfalein retention in the blood stream after one hour, and at the conclusion of the period of intravenous amino acid administration there was only a 12 per cent one-hour retention. The other patients exhibited no significant changes in bromsulfalein excretion.

(e) Effect on other blood constituents: The blood count, nonprotein nitrogen, sedimentation rate, and ieteric indices exhibited no significant changes during the course, or at the conclusion of our period of study. As has been noted, clinical jaundice had subsided in all these patients before the study was begun.

#### SUMMARY

1. A study of the effects of the intravenous administration of amino acids to five patients with cirrhosis of the liver is presented.

2. Clinically, one patient exhibited great general improvement; three exhibited moderate general improvement, and one remained unchanged.

3. The most striking result was shrinkage in the size of the liver and spleen, possibly attributable to resorption of intracellular fat in the liver, with circulatory readjustments secondary to the improved portal circulation resulting in decrease in splenic congestion.

4. Peripheral edema subsided in all patients exhibiting it within two weeks after the initiation of amino acid therapy.

5. Laboratory data accumulated during a preliminary period of observation and at the conclusion of a period of amino acid administration indicated an improvement in glucose tolerance and in hippuric acid synthesis in all patients, and an improvement in bromsulfalein excretion in one patient.

6. Further study and corroboration is needed to determine the degree of benefit obtainable by the use of amino acids in cirrhosis of the liver.

We wish to thank Mr. Raymond L. Charles, chief technician, for his valuable assistance.

The amino acids solution used for this investigation was Amino Acids Parenteral Stearns, supplied through the courtesy of Frederick Stearns & Company, Detroit, Mich.

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# LABORATORY METHODS

## GENERAL

### A STUDY OF THE PHENOMENON OF ERYTHROCYTE SEDIMENTATION\*

#### II. THE ESTABLISHMENT OF A RELIABLE MEASUREMENT OF THE PHENOMENON— ITS REPRODUCIBILITY AND LIMITATIONS

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IN A PREVIOUS publication<sup>1</sup> literary evidence of the source of my conception of the status of sedimentation was advanced. It early became evident that choice or development of a suitable technique for the measurement of sedimentation would be a difficult task indeed since there were and still are in use a multiplicity of techniques, many quite different.

The horse was chosen as the experimental subject since large samples of blood could be procured with ease and at fairly frequent intervals. Agglutination of equine red cells and thus sedimentation are very rapid even in healthy subjects. Probably because of its natural rapidity, separation of equine corpuscles seems somewhat more sensitive to such physiologic factors as fear and hard work and such technical factors as delay, degree of agitation, and anticoagulation. It is felt that this greater sensitivity is a desirable quality because factors which are responsible for variations in sedimentation are more easily discerned and their control is more clearly demonstrated.

Many hundreds of tests were conducted comparing many different techniques and modifications of techniques until finally one was observed that would yield quite comparable results upon repetition, and, since measurement was continuous, was thought to be reasonably complete. With the exception of a description of the technique finally obtained and the suggestion of a more practical "field" technique, together with evidence of the reproducibility and limitations of each, inclusion of the various results of this labor hardly seems necessary here.

#### EQUIPMENT

For collecting bottles ordinary ½ ounce homeopathic vials are measured and scratched at 5 c.c. and 10 c.c. levels. Corks are paraffined. The desired quantity of anticoagulant is added to each vial by first preparing a weak solution of the chosen anticoagulant, adding the volume necessary to each vial and allowing the water to evaporate in the incubator. For routine work a solution of 1.2 per cent ammonium oxalate and 0.8 per cent potassium oxalate is prepared in dis-

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tilled water. One cubic centimeter of this solution when evaporated leaves 12 mg. of ammonium oxalate and 8 mg. of potassium oxalate and is suitable for 10 c.c. of blood—0.5 c.c. of this solution for 5 c.c. of blood. An ample reserve of previously prepared vials is kept on hand.

A sharp clean dry 15 gauge 2 inch hypodermic needle and a clean dry glass syringe of the capacity most suitable are usually employed.

Pledgets and some suitable disinfectant for preparing the area for venipuncture are necessary.

An incubator regulated at body temperature is utilized.

Wintrobe modified hematocrit tubes<sup>2</sup> are employed routinely.

For filling the tubes routinely 18 gauge 4½ inch spinal puncture needles, with round hubs and with the tip ground flat, are used. All filling needles are fitted with rubber medicine dropper bulbs of fairly uniform consistency and capacity. Sixteen gauge 6 inch needles have been more recently used.

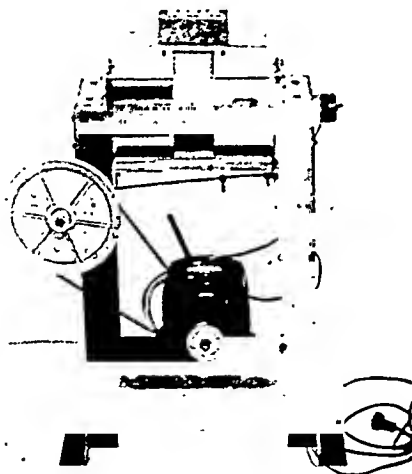


Fig. 1.—Shaking apparatus.

For shaking the blood sample previous to filling the Wintrobe tube a mechanical agitator driven by a variable speed motor has been made. The rack for holding the collecting vials is suspended from four 7¾ inch spring steel arms. This rack is driven over a distance of ¾ inch by a spring steel arm fastened to the bottom of the rack and to a balanced eccentric at the end of a jackshaft. At present the speed of the motor is adjusted to move the rack approximately 564 strokes per minute (Fig. 1).

An automatic recording device employing continuous photography (reported in a previous publication<sup>3</sup>) was made, utilizing the fact that cells cut out most of the light coming through the Wintrobe tube while plasma does not. An image of a very narrow vertical portion of a blood-filled tube is transferred by a photographic lens onto sensitized paper attached to a revolving drum. As the cells settle out more of the sensitized paper is exposed. Results with this apparatus are illustrated in Fig. 3.

Eastman P.M.C. No. 1 normal bromide paper of single weight is used routinely. For developing and fixing solutions those used for ordinary x-ray work are very satisfactory.

A millimeter rule and a simple cardboard "trigonograph" (Chemical Rubber Company) are of great assistance.

The centrifuge employed is an International No. 1 type SB with a head that accommodates eight 15 c.c. conical centrifuge tube holders. The distance from the center of the shaft of the centrifuge to the center of each trunnion ring is  $3\frac{1}{2}$  inches. The speed when the rheostat arm is set at mark "17" is approximately 3,000 r.p.m. with 6 filled Wintrobe tubes. Rubber cushions cut from corks are made for the bottom of the centrifuge tube holders. Side cushions are made from 3 inch lengths of  $\frac{1}{8}$  inch wall,  $\frac{3}{8}$  inch bore rubber tubing.

A rack for placing tubes in a vertical position for the "field" technique was made by machining parallel V-shaped grooves on the side of a brass casting. These grooves can be rendered perpendicular by adjustable legs, using as a guide a special plumb bob fastened to one end of the machined casting. The sides of the tubes are held against the sides of the V-groove by spring brass clips (Fig. 2).

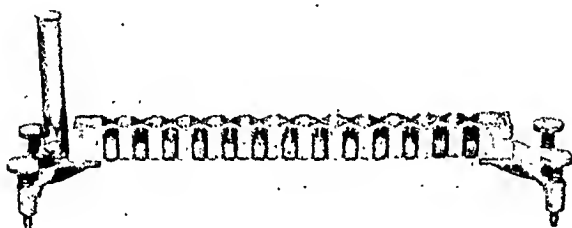


Fig. 2.—Perpendicular tube rack.

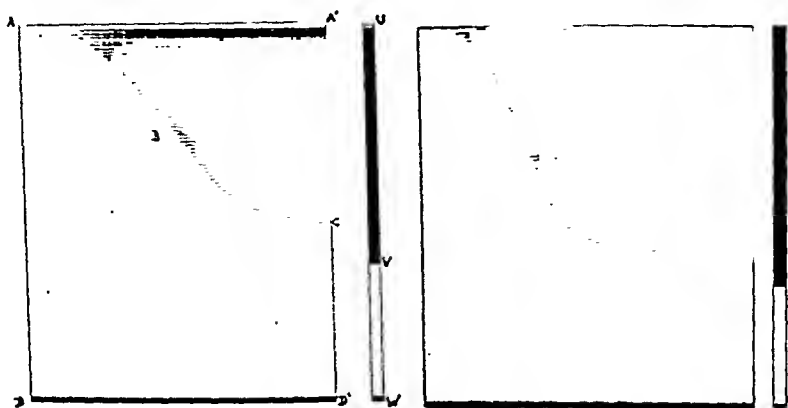
#### DESCRIPTION OF TECHNIQUE

When all minor details pertaining to equipment, restraint, disinfection of the area, etc., have been taken into consideration, the jugular vein of the animal chosen is raised by thumb pressure. As soon as the needle is in the vein, this pressure is completely released and, after a second's hesitation to allow the establishment of free flow in the vein, the desired quantity of blood is withdrawn into a syringe. After obtaining this desired quantity of blood in the syringe, the collecting vial (or vials as the case may be) is immediately filled to the necessary mark, corked, and is thoroughly agitated immediately to mix the blood and anticoagulant. Of course, the syringe must be washed before clotting can take place.

The collecting vial (or vials) is then placed in a quiet place until the cells have settled, which with horse blood usually requires anywhere from five to twenty or twenty-five minutes. Next the vial (or vials) is placed in the mechanical shaker for fifteen minutes. While the shaker is still in motion, the cork is taken out, a quantity of blood is removed from the bottom of the vial by the filling needle and bulb, and a Wintrobe tube is carefully filled to the "100 mm." mark *avoiding forceful filling* and bubbles. The tube is then placed in the tube holder of the automatic recorder and the shutter is opened. Recording is allowed to take place for whatever time the sample indicates—usually thirty minutes from the time of filling the tube. If settling is not fairly complete, a longer time

can be chosen but routinely this is rarely necessary. After recording, the same sample can be centrifuged (about 3,000 r.p.m. per thirty minutes), and the results are either photographed on the same record or read off directly from the tube.

After the picture is exposed, developed, and dry, measurements of whatever sort desired can be made.



Ex. 2.

Fig. 3.—Examples of results as obtained with the photographic sedimentation recorder.

Examples 1 and 2 are results obtained by the photographic recorder, example 1 being labeled for explanatory purposes (Fig. 3):

DD' is the top of the dark heavy line and represents the bottom of the column of blood. The length of DD' (or AA') represents the time of exposure.

AD represents the height of the column of blood (100 mm.) at the start of the measurement when the cells are dispersed throughout the plasma.

The dark area ABCA' represents the exposed area of the graph brought about by the increasing amount of light passing through the plasma as the cells settle out.

The light area ABCD'D represents the unexposed area brought about by the presence of cells through which the light cannot pass.

AB represents the beginning and gradually accelerated descent of the line of demarcation between cells and plasma.

BC represents the gradually retarded descent of the line of demarcation between the cells and plasma brought about by the gradually increasing effect of heaping of cells in the lower portion of the tube.

B represents the theoretical point of change between the gradually accelerated descent (AB) and the gradually retarded descent (BC) of the line of demarcation between cells and plasma.

Sigmoid curve ABC then represents a graphic record of the descent of the line of demarcation between cells and plasma for a given time.

The small light horizontal lines in area ABCA' represent the millimeter divisions on the tube.

The small vertical lines in area ABCA' occur at intervals of approximately 1.02 minutes as the apparatus is now run.

The narrow dark vertical lines preceding and following exposure of the sedimenting and centrifuged sample are made by slight exposure of the sensitized paper when the tube holder is empty. These are essentially not necessary but are handy.

UW represents the height of the column of blood (100 mm.).

UV represents the height of the column of plasma after centrifugation (volume percentage of plasma).

VW represents the height of the column of cells after centrifugation (volume percentage of cells).

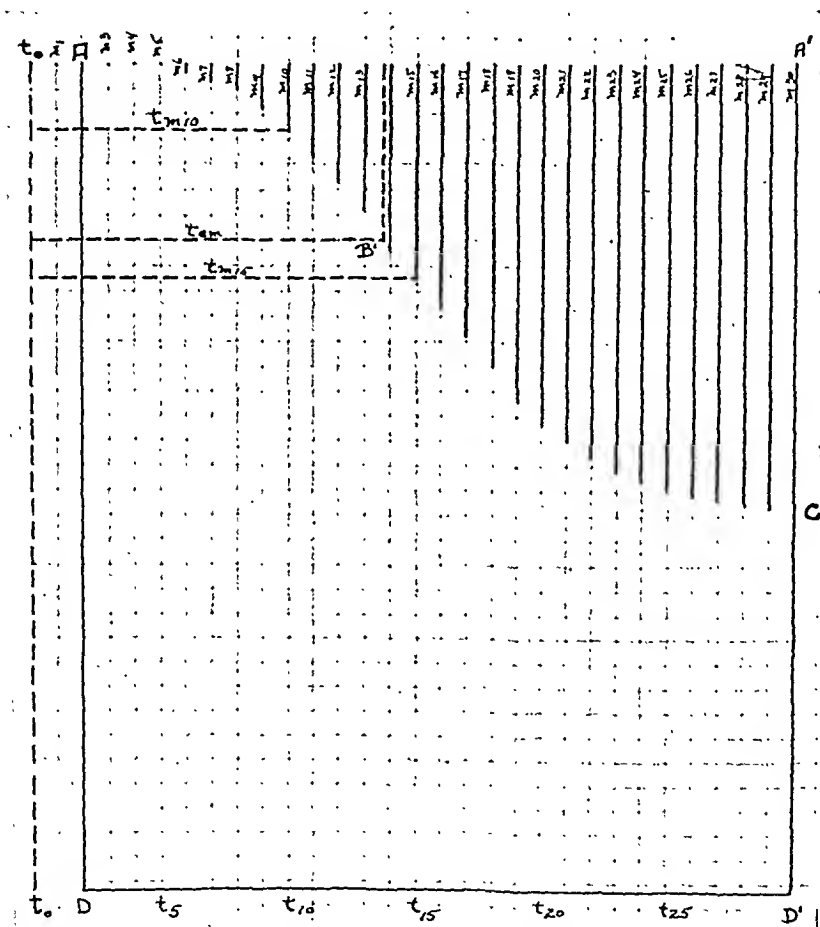


Fig. 4.—EX. 1 of Fig. 3 plotted graphically.

To clarify some of the measurements made from the afore-mentioned photographs the following graph of example 1 is presented in Fig. 4:

1. Line  $t_0-t_0$  is located by subtracting the time of filling the tube from the time that the exposure is completed, multiplying by 3 (since 3 mm. are the equivalent of one minute on the photograph), and then laying this distance off from  $A'D'$ , the end of the exposure. Line  $t_0-t_0$  then represents the time of filling of the tube. In some of these examples  $t_0-t_0$  is purposely quite a distance

from AD, this to separate  $t_0-t_0$  from AD to make a clearer graph. In practice  $t_0-t_0$  is usually not more than fifteen to thirty seconds from AD.

2. Times ( $t$ ) are measured in minutes from  $t_0-t_0$  as  $t_1, t_2, t_7$ , etc., subnumbers representing the number of minutes from  $t_0-t_0$  (3 mm. of horizontal distance equal one minute).

3. Perpendicular distances ( $m$ ) from line  $AA'$  to curve ABC are measured in millimeters as  $m_2, m_3$ , and  $m_7$ , etc., the subnumbers representing times in minutes from  $t_0-t_0$  that these distances are measured.  $m$  is affected by variations in filling the tube to the "100 mm." mark and by variations in image size. However, these are usually very small and can be allowed for during measurement.

4.  $B'$  is the estimated point of change between the vertical flexion ( $AB'$ ) of curve  $AB'C$  and the horizontal flexion ( $B'C$ ). With care and experience this point can be very accurately adjudged, especially if the curve is tilted in such a way that the eye can follow an imaginary tangent to the steepest portion of curve  $AB'C$ . Assistance in judging this point is also furnished by applying a straightedge as a tangent.

5.  $em$  is the perpendicular distance from  $AA'$  to  $B'$ . This measurement is subject to the same variations as  $m$ . In Ex. 1: 21 mm.

6.  $t_{em}$  is the horizontal distance from  $t_0-t_0$  to  $B'$  divided by 3 (since a horizontal distance of 3 mm. on the photograph represents one minute) and expressed as minutes. Ex.: 13.8 minutes.

7.  $\angle_g$  is the least angle between the apparently steepest slope of curve  $AB'C$  and  $m$ . This angle can be measured by lining up AD with one side of a trigonograph and moving the free arm of the trigonograph until it lies tangent to the apparently steepest slope of curve  $AB'C$ , this being very closely related to the A side of  $B'$ . Considerable error can be observed if the free arm of the trigonograph is lined up with too great a distance on curve  $AB'$ . Careful and close scanning will allow use of as little as 1 to 2 mm. on curve  $AB'$  as a tangent to the free arm. Ex.:  $32^\circ 20'$ .

8.  $I_{em}$  is an index calculated by the formula: 
$$\frac{em}{t_{em} \times 3}.$$

Ex.:  $\frac{21}{41.5}$  or 0.51.

If one-minute readings of the height of the column of cells are made, as for instance in Ex. 1:

$t_0 = 100$  mm.  
 $t_1 = 100$  mm.  
 $t_2 = 100$  mm.  
 $t_3 = 100$  mm.  
 $t_4 = 100$  mm.  
 $t_5 = 100$  mm.  
 $t_6 = 99$  mm.  
 $t_7 = 98$  mm.

$t_8 = 97$  mm.  
 $t_9 = 94\frac{1}{2}$  mm.  
 $t_{10} = 92$  mm.  
 $t_{11} = 89$  mm.  
 $t_{12} = 86$  mm.  
 $t_{13} = 82\frac{1}{2}$  mm.  
 $t_{14} = 78$  mm.  
 $t_{15} = 74\frac{1}{2}$  mm.

$t_{16} = 71$  mm.  
 $t_{17} = 67\frac{1}{2}$  mm.  
 $t_{18} = 64$  mm.  
 $t_{19} = 60$  mm.  
 $t_{20} = 57$  mm.  
 $t_{21} = 55$  mm.  
 $t_{22} = 53$  mm.  
 $t_{23}$  etc.

and if these are subtracted from the height of the column of blood (usually 100 mm.), values of  $m$  will be:

$t_0 = 0$  mm.  
 $t_1 = 0$  mm.  
 $t_2 = 0$  mm.  
 $t_3 = 0$  mm.  
 $t_4 = 0$  mm.  
 $t_5 = 0$  mm.  
 $t_6 = 1$  mm.  
 $t_7 = 2$  mm.

$t_8 = 3$  mm.  
 $t_9 = 5.5$  mm.  
 $t_{10} = 8$  mm.  
 $t_{11} = 11$  mm.  
 $t_{12} = 14$  mm.  
 $t_{13} = 17.5$  mm.  
 $t_{14} = 22$  mm.  
 $t_{15} = 25.5$  mm.

$t_{16} = 29$  mm.  
 $t_{17} = 32.5$  mm.  
 $t_{18} = 36$  mm.  
 $t_{19} = 40$  mm.  
 $t_{20} = 43$  mm.  
 $t_{21} = 45$  mm.  
 $t_{22} = 47$  mm.  
 $t_{23}$  etc.

To continue the definitions:

9.  $M_1$  is the maximum difference in millimeters between any two consecutive minute readings of  $m$ . In the above example,  $4\frac{1}{2}$  mm.

10.  $t_{M_1}$  symbolizes the times in minutes from  $t_0$ - $t_0$  between which  $M_1$  occurs. In the above example, thirteen to fourteen minutes.

11.  $I_{M_1}$  represents an index range calculated by dividing the values of  $m$  between which  $M_1$  occurs by their corresponding times multiplied by 3, since 3 mm. is the equivalent of one minute. Ex.:  $\frac{17.5}{39} - \frac{22}{42}$  or  $0.45 - 0.52$ .

12.  $\angle_{M_1}$  is measured by calculating on the trigonograph the angle opposite the horizontal side of a right triangle having  $M_1$  as the perpendicular side and 3 mm. (one minute) as the horizontal side. Ex.:  $33^\circ 55'$ .

13.  $M_5$  is the maximum difference in millimeters between any two consecutive five-minute readings of  $m$  starting at  $t_0$ - $t_0$ ; i.e., the maximum five-minute fall of the line of demarcation between cells and plasma. Ex.: 17.5 mm.

14.  $t_{M_5}$  symbolizes the times in minutes from  $t_0$ - $t_0$  between which  $M_5$  occurs. Ex.: ten to fifteen minutes, fifteen to twenty minutes.

15.  $I_{M_5}$  represents an index range calculated by dividing the values of  $m$  between which  $M_5$  occurs by their corresponding times multiplied by 3, since 3 mm. are the equivalent of one minute. Ex.: 0.27 to 0.47, 0.57 to 0.72.

16.  $\angle_{M_5}$  is equivalent to the angle opposite the horizontal side of a right triangle having  $M_5$  as the perpendicular side and 15 mm. (five minutes) as the horizontal side. Ex.:  $40^\circ 40'$ .

If readings are made of  $m$  every five minutes, starting at times other than  $t_0$ - $t_0$ , we find differences in  $M_5$ ,  $I_{M_5}$ , and  $\angle_{M_5}$ :

| Time      | $M_5$               | $t_{M_5}$   | $I_{M_5}$   | $\angle_{M_5}$ |
|-----------|---------------------|---|---|----------------|
| $t_{0-5}$ | $17\frac{1}{2}$ mm. | $\left\{ \begin{array}{l} 15-20 \text{ min.} \\ 20-25 \text{ min.} \end{array} \right.$ | $\left\{ \begin{array}{l} 0.18-0.43 \\ 0.43-0.57 \end{array} \right.$ | $40^\circ 40'$ |
| $t_{0-4}$ | 18 mm.              | 15-20 min.  | 0.24-0.48   | $40^\circ$     |
| $t_{0-3}$ | $18\frac{1}{2}$ mm. | 15-20 min.  | 0.31-0.54   | $39^\circ 10'$ |
| $t_{0-2}$ | $18\frac{1}{2}$ mm. | 15-20 min.  | 0.39-0.5  | $39^\circ 10'$ |
| $t_{0-1}$ | 18 mm.              | 15-20 min.  | 0.49-0.67   | $40^\circ$     |
| $t_0$     | $17\frac{1}{2}$ mm. | $\left\{ \begin{array}{l} 10-15 \text{ min.} \\ 15-20 \text{ min.} \end{array} \right.$ | $\left\{ \begin{array}{l} 0.27-0.57 \\ 0.57-0.72 \end{array} \right.$ | $40^\circ 40'$ |
| $t_{0+1}$ | 18 mm.              | 10-15 min.  | 0.37-0.64   | $40^\circ$     |
| $t_{0+2}$ | $18\frac{1}{2}$ mm. | 10-15 min.  | 0.47-0.72   | $39^\circ 10'$ |
| $t_{0+3}$ | $18\frac{1}{2}$ mm. | 10-15 min.  | 0.58-0.80   | $39^\circ 10'$ |
| $t_{0+4}$ | 18 mm.              | 10-15 min.  | 0.73-0.89   | $40^\circ$     |
| $t_{0+5}$ | $17\frac{1}{2}$ mm. | $\left\{ \begin{array}{l} 5-10 \text{ min.} \\ 10-15 \text{ min.} \end{array} \right.$  | $\left\{ \begin{array}{l} 0.53-0.85 \\ 0.85-0.96 \end{array} \right.$ | $40^\circ 40'$ |

To continue the definitions:

17.  $GM_5$  is the largest maximum difference in millimeters between any two consecutive five-minute readings of  $m$  beginning anywhere between  $t_{0-5}$  and  $t_{0+5}$ . Ex.:  $18\frac{1}{2}$  mm.

18.  $SM_5$  is the smallest maximum difference in millimeters between any two consecutive five-minute readings of  $m$  beginning anywhere between  $t_{0-5}$  and  $t_{0+5}$ . Ex.:  $17\frac{1}{2}$  mm.



19.  $t_{GM_s}$  represents the times in minutes between which  $GM_s$  occurs. Ex.: 10-15 and 15-20 min.

20.  $t_{SM_s}$  represents the times in minutes between which  $SM_s$  occurs. Ex.: 10-15 and 15-20 min.

21.  $I_{GM_s}$  represents an index range calculated by dividing the values of  $m$  between which  $GM_s$  occurs by their corresponding times multiplied by 3, since 3 mm. are equivalent to one minute. Ex.: 0.31 to 0.8.

22.  $I_{SM_s}$  represents an index range calculated by dividing the values of  $m$  between which  $SM_s$  occurs by their corresponding times multiplied by 3, since 3 mm. are equivalent to one minute. Ex.: 0.27 to 0.72.

23.  $\angle_{GM_s}$  is the angle opposite the horizontal side of a right triangle having  $GM_s$  as the perpendicular side and 15 mm. (five minutes) as the horizontal side. Ex.:  $39^\circ 10'$ .

24.  $\angle_{SM_s}$  is the angle opposite the horizontal side of a right triangle having  $SM_s$  as the perpendicular side and 15 mm. as the horizontal side. Ex.:  $40^\circ 40'$ .

25.  $\overline{M}_s$  is the mean of the  $M_s$  calculations from  $t_0$ - $t_{0.24}$ . Ex.: 18.1 mm.

26.  $\angle_{\overline{M}_s}$  is the angle opposite the horizontal side of a right triangle having  $\overline{M}_s$  as the perpendicular side and 15 mm. (five minutes) as the horizontal side. Ex.:  $39^\circ 50'$ .

27.  $V\%$  is the centrifuged volume per cent of cellular material.

A chart of measurements of examples can now be presented:

|        | $V\%$            | em                        | $t_{em}$                | $I_{em}$        | $\angle_{\overline{M}_s}$ |
|--------|------------------|---------------------------|-------------------------|-----------------|---------------------------|
| Ex. 1. | 36-              | 21 mm.                    | 13.8 min.               | 0.51            | $39^\circ 20'$            |
| Ex. 2. | 31½              | 34 mm.                    | 10.0 min.               | 1.10            | $18^\circ 10'$            |
|        | $M_s$            | $t_{M_s}$                 | $I_{M_s}$               | $\angle_{M_s}$  |                           |
| Ex. 1. | 4.5 mm.          | 13-14 min.                | 0.45-0.52               | $33^\circ 50'$  |                           |
| Ex. 2. | 9 mm.            | 9-10 min.                 | 0.93-1.13               | $18^\circ 20'$  |                           |
|        | $M_s$            | $t_{M_s}$                 | $I_{M_s}$               | $\angle_{M_s}$  |                           |
| Ex. 1. | 17.5 mm.         | {10-15 min.<br>15-20 min. | {0.27-0.57<br>0.57-0.72 | $40^\circ 40'$  |                           |
| Ex. 2. | 21 mm.           | 5-10 min.                 | 0.20-1.13               | $26^\circ 00'$  |                           |
|        | $GM_s$           | $t_{GM_s}$                | $I_{GM_s}$              | $\angle_{GM_s}$ |                           |
| Ex. 1. | 18.5 mm.         | {10-15 min.<br>15-20 min. | 0.31-0.80               | $39^\circ 10'$  |                           |
| Ex. 2. | 24 mm.           | {5-10 min.<br>10-15 min.  | 0.23-1.57               | $24^\circ 10'$  |                           |
|        | $SM_s$           | $t_{SM_s}$                | $I_{SM_s}$              | $\angle_{SM_s}$ |                           |
| Ex. 1. | 17.5 mm.         | {10-15 min.<br>15-20 min. | {0.27-0.57<br>0.57-0.72 | $40^\circ 40'$  |                           |
| Ex. 2. | 29 mm.           | {5-10 min.<br>10-15 min.  | 0.83-1.80               | $27^\circ 30'$  |                           |
|        | $\overline{M}_s$ | $\angle_{\overline{M}_s}$ |                         |                 |                           |
| Ex. 1. | 18.1             | $39^\circ 50'$            |                         |                 |                           |
| Ex. 2. | 32.0             | $25^\circ 20'$            |                         |                 |                           |

## COMPARISONS

Several features of the foregoing measurements of examples are quite noticeable:

1. Estimations of  $em$  are in all probability quite accurate because  $t_{em}$  in each case lies between the limits of  $t_{M_1}$  and also  $t_{M_5}$ ,  $t_{GM_5}$  and  $t_{SM_5}$ .
2. Since the above is true, calculations of  $I_{em}$  are expected to lie and lie within the ranges of  $I_{M_1}$ ,  $I_{M_5}$ ,  $I_{GM_5}$ , and  $I_{SM_5}$ .
3. The nearer  $t_{em}$  approaches either of the limits of  $t_{M_1}$ , etc., the closer  $I_{em}$  approaches the corresponding limit of  $I_{M_1}$ , etc. Such would be expected.
4.  $\angle_s^e$  are less than  $\angle_{M_1}^s$ ,  $\angle_{M_5}^s$ ,  $\angle_{GM_5}^s$ ,  $\angle_{SM_5}^s$ , and  $\angle_{\overline{M_5}}^s$  and upon examination of Fig. 5 rightfully should be.

When one considers that  $B'$  represents in reality not a point on a straight line between  $A$  and  $C$ , but the point of change of a shallow sigmoid curve between  $A$  and  $C$ , then one will realize that the least angle between curve  $AB'$  and a perpendicular line from  $B'$  to  $AA'$  will be less than angle  $ACA'$ . This also applies to estimates of angles derived from  $M_5$ ,  $GM_5$ ,  $SM_5$ , and  $\overline{M_5}$ . On first thought one would say then that the nearer the angles estimated from the differences between interval readings of  $m$  approach  $\angle_s^e$ , the more accurate they are. One might also say that the longer the interval between readings of  $m$ , the more deceiving will be the plotted curve and the more inaccurate the estimation of angles, maximum falls, indexes, etc.

Since smaller measurements are generally accompanied by larger possible errors,  $M_1$  is thought to be accompanied by greater possible error than measurements of  $M_5$ . To illustrate this, consider the effect on  $M_1$  and  $M_5$  that a fifteen-second variation in  $t_0$  will cause. Fifteen seconds is  $\frac{1}{4}$  of one minute but only  $\frac{1}{20}$  of five minutes. Also consider the effect on  $M_1$  and  $M_5$  that 0.5 mm. variation will cause. In example 1, 0.5 mm. is  $\frac{1}{9}$  of 4.5 mm. but only  $\frac{1}{35}$  of 17.5 mm. Is it unreasonable to assume then that  $M_1$  is subject to greater variations than  $M_5$  and  $t_{M_5}$  in actual measurement? Also is it unreasonable to assume that errors in measurement of  $M_1$  will produce greater error in estimation of  $\angle_{M_1}$  than error in measurement of  $M_5$  will produce error in estimation of  $\angle_{M_5}$ ? Is it correct to assume then that, although  $\angle_{M_1}$  and  $\angle_{GM_5}$  more closely approach  $\angle_s^e$ , they are more susceptible to error as estimates of  $\angle_s^e$  than  $\angle_{M_5}$ ? In final analysis  $\angle_{GM_5}$  is not derived from the maximum five-minute falls where measurement is started at  $t_0$  but at times other than  $t_0$ , sometimes even at  $t_{0+4}$ . The same applies to  $\angle_{SM_5}^s$  and  $\angle_{\overline{M_5}}^s$ . Their proximity to  $\angle_s^e$  does not necessarily denote the degree of their accuracy.

Would it be in error to assume that if measurements of  $M_5$  and  $\angle_{M_5}^s$  on replicate aliquots are possible with a fair degree of accuracy these would at least roughly represent the separation of formed and fluid elements of the blood?

Evidence is convincing to me at least that the best estimate of separation is the continuous measurement of the line of demarcation between cells and plasma with estimation of the point of change in the resulting sigmoid curve. It is felt that if measurements of the point of change are to express even partially the important aspects of the curve they must include the time at which this point of change occurs ( $t_{em}$ ), the amount of separation before the point of change takes place ( $em$ ), and the rate of speed of separation immediately before the point of change (expressed as  $\angle_{\pi}$ ).

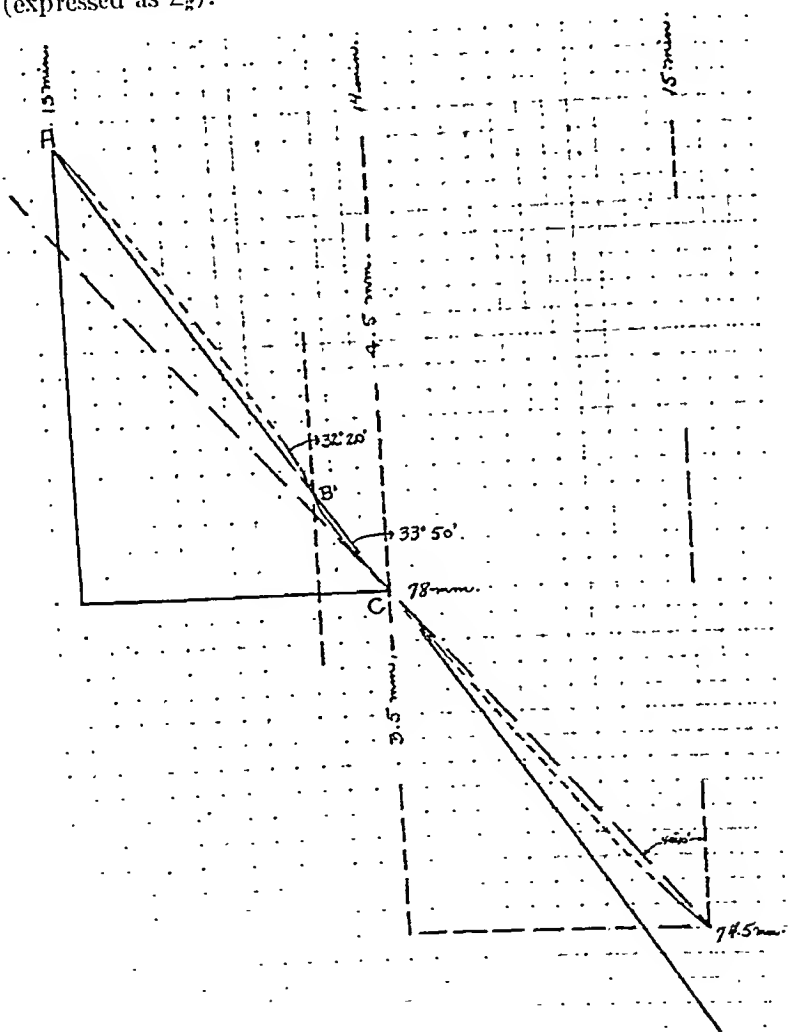


Fig. 5.—Ex. 1 of Fig. 3—graph of area near B' (enlarged).

However, with practitioners, simplicity of technique is the keynote and often a certain degree of accuracy must be sacrificed for practicability. In view of this a *more practical technique* is devised, results of which will be reported in many instances along with results of the photographic technique and in many instances alone.

With this more practical "field" technique the blood sample can be drawn and divided in the same manner as described under the photographic technique. The vials are allowed to remain at room temperature until settled, are agitated as before, and the Wintrobe tubes are filled as previously described. However,

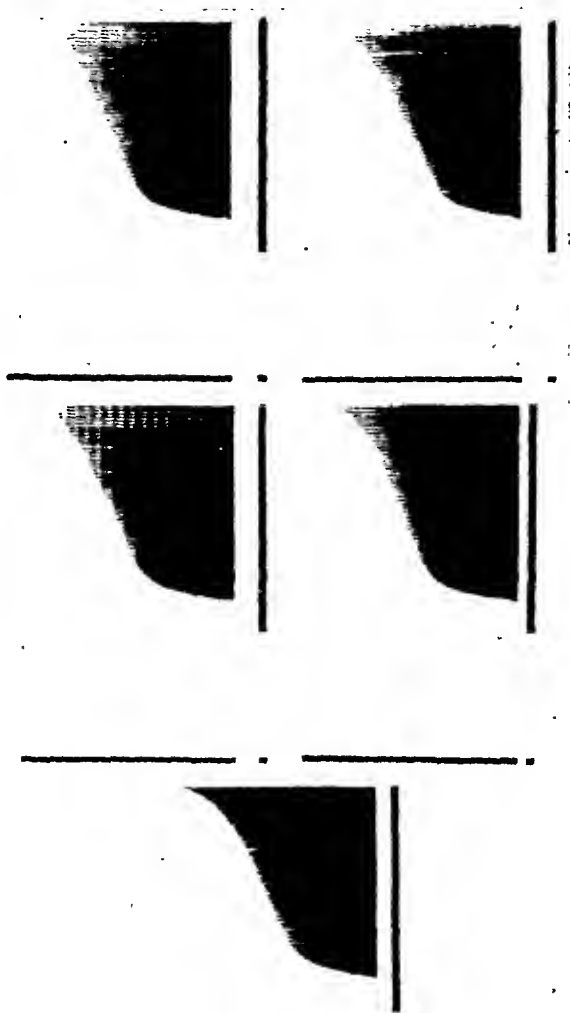


Fig. 8.—Evidence of the reproducibility of sedimentation curves as obtained from five aliquots of the same sample of blood.

instead of being put into the photographic recorder each tube is placed into the perpendicular slots of the previously described rack, and the line of demarcation between cells and plasma read five minutes after filling and at five-minute intervals thereafter until maximum settling is passed. The greatest difference in millimeters between any two consecutive five-minute readings is recorded.  $t_{M_5}$  and  $\angle_{M_5}$  are determined. These three measurements can be considered as locat-

ing quite roughly, to be sure, the point of change between increasing and decreasing rate of descent of the line of demarcation between cells and plasma and the speed of separation.

## REPRODUCIBILITY

Now it seems logical to test whether several aliquots of the same sample of blood will yield comparable results.

The reproducibility of the photographic technique is demonstrated by Fig. 6, representing curves of sedimentation in Wintrobe tubes filled from five 10 c.c. aliquots of the same sample of blood. Data from these are as follows:

| Ex. No. | em       | $t_{em}$  | $I_{em}$ | $\angle_{\Sigma}^s$ | V%   |
|---------|----------|-----------|----------|---------------------|------|
| 1       | 29 mm.   | 9.2 min.  | 1.05     | 22° 50'             | 35.3 |
| 2       | 29 mm.   | 10.0 min. | 0.97     | 22° 10'             | 35.3 |
| 3       | 29 mm.   | 9.0 min.  | 1.07     | 22° 30'             | 35.5 |
| 4       | 30 mm.   | 9.2 min.  | 1.09     | 22° 00'             | 36   |
| 5       | 30 mm.   | 10.0 min. | 1.0      | 21° 30'             | 35.7 |
| Mean    | 29.4 mm. | 9.48 min. |          | 22° 24'             | 35.6 |

| Ex. No. | $M_s$    | $t_{M_s}$ | $I_{M_s}$ | $\angle_{M_s}^s$ |
|---------|----------|-----------|-----------|------------------|
| 1       | 27 mm.   | 5-10 min. | 0.53-1.17 | 29° 30'          |
| 2       | 25 mm.   | 5-10 min. | 0.47-1.07 | 31° 20'          |
| 3       | 27 mm.   | 5-10 min. | 0.67-1.23 | 29° 30'          |
| 4       | 28 mm.   | 5-10 min. | 0.47-1.17 | 28° 30'          |
| 5       | 27 mm.   | 5-10 min. | 0.27-1.03 | 29° 30'          |
| Mean    | 26.8 mm. | 5-10 min. |           | 30° 04'          |

Since the determinants of  $I_{em}$  are em and  $t_{em}$ ,  $I_{em}$  is thought superfluous. In addition  $I_{em}$  may be deceiving, since it expresses a ratio and could represent other values of em and  $t_{em}$  than those actually measured; for instance, an  $I_{em}$  of 1.0 might represent, as in Ex. 5, an em of 30 mm. and a  $t_{em}$  of ten minutes (30 mm.), but it might also represent an em of 15 mm. and a  $t_{em}$  of five minutes (15 mm.) or any other combination where em in millimeters is equal to  $t_{em}$  in millimeters. For this reason its use is dropped. Since  $I_{M_s}$  merely places rough limits to  $I_{em}$ , it too is dropped. An index of maximum "rise" ("fall" in this means of expression) over "run," which has been employed extensively in human techniques, has been neglected in this work chiefly because  $\angle_{M_s}$  in reality is identical with this index. Since  $\angle_{\Sigma}$  is felt to represent more accurately the actual "speed" of settling and since  $\angle_{\Sigma}$  is best measured and expressed in terms of degrees and fractions thereof, for comparative purposes "speed" in the "field" technique is designated in this same terminology, keeping in mind, of course, that  $\angle_{\Sigma}$  must be less than  $\angle_{M_s}$ .

Agreement in repetition of the "field" technique can be demonstrated by the following examples derived from measurements with Wintrobe tubes filled from five 10 c.c. aliquots of the same sample of blood:

| Aliquot No. | $M_s$  | $t_{M_s}$  | $\angle_{M_s}^s$ | V%    |
|-------------|--------|------------|------------------|-------|
| 1           | 24 mm. | 15-20 min. | 32° 20'          | 34.5  |
| 2           | 23 mm. | 15-20 min. | 33° 30'          | 34.5+ |
| 3           | 24 mm. | 15-20 min. | 32° 20'          | 35.0- |
| 4           | 25 mm. | 15-20 min. | 31° 20'          | 34.0+ |
| 5           | 25 mm. | 15-20 min. | 31° 20'          | 34.5+ |

Occasionally one will observe very close figures of  $M_s$ ,  $\angle^s M_s$ , and  $V\%$ , but  $t_{M_s}$  will vary as in this example:

| Aliquot No. | $M_s$ . | $t_{M_s}$  | $\angle^s M_s$ | $V\%$ |
|-------------|---------|------------|----------------|-------|
| 1           | 19 mm.  | 15-20 min. | 38° 50'        | 41.0  |
| 2           | 20 mm.  | 15-20 min. | 37° 20'        | 41.0- |
| 3           | 19 mm.  | 20-25 min. | 38° 50'        | 41.0+ |
| 4           | 18 mm.  | 20-25 min. | 40° 10'        | 41.0- |
| 5           | 18 mm.  | 20-25 min. | 40° 10'        | 41.0+ |

This is not thought to be a serious error and the apparent reasons for such will be discussed in a future article under the heading of agitation. Further evidence of the reproducibility of the "field" technique will be observed as one continues through the remainder of this series.

In the next two publications of this series technical sources of variation will be demonstrated and discussed.

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## OBJECTIVE METHOD FOR DEMONSTRATION OF LOCAL RIGIDITY OF THE ABDOMINAL WALL\*

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LOCAL rigidity of the abdominal wall is a well-known symptom of some abdominal diseases. This phenomenon is commonly thought to be due to the increase in tension of some abdominal muscles and is detected by comparative palpation of symmetrical parts of the abdominal wall. However, the tactile sense is not reliable; even experienced observers in palpating the abdominal wall may get contradictory results. The assumption that local rigidity is caused by differences between tensions of both sides of the abdominal wall has been challenged by Vaughan.<sup>1</sup> According to him, such differences can be observed only if the two parts are palpated one after the other. Vaughan asserts that palpation of the diseased side elicits a contraction of the abdominal muscles and this contraction occurs equally on both sides. Thus, if both sides are being palpated simultaneously by both hands, there is no difference observed except in

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the presence of a palpable mass on the affected side. Work,<sup>2</sup> too, questions the occurrence of differences between the tensions of the two sides of the abdominal wall. However, at least the possibility of such differences is shown by the example of the diaphragm. In inflammatory conditions of the abdomen, when the lesion is close to one side of the diaphragm, immobilization of this side of the diaphragm in high position can often be observed by fluoroscopy. Further, in cases of acute appendicitis, Sale<sup>3</sup> found that the right diaphragm was, as a rule, flatter than the left.

To compare the resistance of symmetrical parts of the abdominal wall in an *objective* manner, equal forces acting in symmetrical directions and upon equal areas should be applied, and the depth of the depressions measured. However, the movements of the abdominal wall during breathing interfere with the making of such measurements. Evidently the measurements should be made in a respiratory pause and on both sides in the same phase of respiration. Kelley<sup>4</sup> described an apparatus which he used to make such measurements. However, in Kelley's method the direction of the force cannot be established accurately, and no provisions are set forth for making the measurements in the same respiratory phase. Work<sup>2</sup> made similar measurements in normal and pathologic cases with an apparatus in which a pair of weights are used to exert forces upon symmetrical parts of the abdominal wall. The whole apparatus rests on the abdominal wall. As the result of his measurements, Work makes the statement that no differences between the tensions of the two sides of the abdominal wall occur. However, if the forces exerted by the cylindrical weights are to be definite, the longitudinal axes of the weights must be kept exactly vertical. Work sets the crossbar of the apparatus in a horizontal position. But the position of the whole apparatus is subject to changes caused by the respiratory movements of the abdominal wall because it seems hardly possible that the subject could hold his breath during the whole time of setting the apparatus by a spirit level and making the measurements. Thus Work's measurements do not seem to prove his assertion that no differences occur between the tensions of the two sides of the abdominal wall.

To demonstrate local rigidities of the abdominal wall in an objective way a new method has been developed which is based upon the detection of differences between the respiratory movements of symmetrical parts of the abdominal wall. In this way, instead of being a disturbing factor, the respiratory displacement of the abdominal wall becomes the object of the measurement. In other words, in making the measurements the force originating from the contraction of the diaphragm is used rather than external forces. The contracting diaphragm acts like a piston producing compression, thus increasing the intra-abdominal pressure during inspiration. If the subject is in an exactly horizontal supine position, the pressure forces acting upon equal areas of symmetrical parts of the abdominal wall are equal and of symmetrical direction. The following discussion shows, namely, that the abdominal cavity can be considered as a closed container filled with a liquid, and that the conditions of equilibrium of this cavity and the pressure forces acting upon its surface are subject to the laws of hydrostatics.

## VALIDITY OF THE LAW OF PASCAL FOR THE ABDOMINAL CAVITY

The law of Pascal states that if only surface forces are acting upon a liquid, the pressure is the same at every point and in every direction. True, the contents of the abdominal cavity are solids. However, a solid body which does not resist bending and is not acted upon by tensile forces behaves statically like a liquid and obeys Pascal's law. This is true for a considerable part of the abdominal organs (stomach, intestines, peritoneal folds) which, when put into an open container, assume its form and present a horizontal surface. The other group of the abdominal organs are solids in every respect and, therefore, do not obey the laws of hydrostatics in their interior. Their presence, however, does not affect the validity of the law of Pascal for the abdominal cavity just as the presence of solid bodies would be of no consequence upon the validity of the same law for a real fluid. Upon the contents of the abdominal cavity, in addition to surface forces originating from the tension of the abdominal walls, a mass force—gravity—is acting. The effect of the latter is shown by pressure differences between various levels of the cavity, all points of the same level having the same pressure. Since differences in specific gravity of the solid organs and of their surroundings are negligible, these organs float freely, hindered in their movements only by peritoneal bands. Under ordinary conditions the bands are loose and the solid organs are acted upon solely by the intra-abdominal pressure. Naturally, these bodies establish equilibrium by exerting exactly the same pressure on their surroundings. Thus, whether or not the abdominal walls are in direct contact with solid organs, no forces other than originating from the abdominal pressure are acting on their internal surface. There are only two exceptions: First, the anterior and posterior abdominal walls may be forced apart by a solid organ during the whole period of respiration or, more often, during deep expiration only. In this case a local increase in pressure will result while the organ itself undergoes elastic deformation. The other exception concerns gas-filled intestinal loops. Such a loop has a mean specific gravity considerably less than that of its surroundings, and thus it is acted upon by a buoyant force. Hence the loop tends to occupy the highest possible position, either stretching the corresponding bands, or protruding the abdominal wall. With these two exceptions, which will be discussed later, the equilibrium positions of the different parts of the abdominal wall in a given position of the subject are determined by the elastic properties of this wall and by the intra-abdominal pressure. They are not influenced by the position of the abdominal organs, such as adjacency to the abdominal wall. The slow respiratory movements of the abdominal wall can be considered as consisting of a continuous series of equilibrium positions. Thus, with the subject in an exactly horizontal supine position, simultaneous respiratory amplitudes of symmetrical parts of the abdominal wall must be equal if the structures of the two sides of the abdominal wall are in every respect alike. On the other hand, any unilateral change may create a difference between simultaneous respiratory amplitudes of the two sides.



# APPARATUS FOR THE OBSERVATION OF DIFFERENCES BETWEEN RESPIRATORY AMPLITUDES

The method to be described involves the use of an apparatus somewhat resembling a large caliper. It has two aluminum legs (about 15 cm. long) connected at the top by a nearly frictionless hinge which allows them to move freely in only one plane. The length of the legs can be increased to about 30 cm. by screwing on extensions. The end of the legs and of the extensions are pointed. A sharp-pointed steel index rod, about 2 cm. long, is attached to the hinge perpendicularly to the plane of the legs. The weight of the apparatus with extensions is 40 Gm. For the examination the subject is placed in a reclining position so that his frontal plane is horizontal, the knees being slightly bent and supported by pillows. The parts to be compared are covered by small pieces of

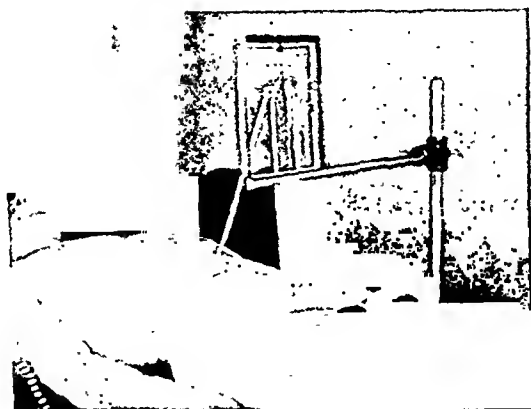


Fig. 1.

adhesive tape and the points of the legs are placed thereon. To the table on which the subject is lying a glass plate, 8 by 20 cm., with reference line in the middle, is attached in such a way that the line is vertical. The instrument is then leaned against the plate, the point of the index rod near the reference line (Fig. 1). The angle between the plate and the plane of the legs should be about  $10^\circ$ , and symmetrical points of the legs should be at equal distance from the glass plate. If the distance between the points examined is about 6 to 7 cm. then the shorter legs should be used; if the distance is about 12 to 15 cm. the extensions are required; the angle between the two legs should be approximately  $25^\circ$ . There is little friction between the glass plate and the index point because the weight of the instrument has only a small component perpendicular to the glass plate. During breathing the point of the index rod moves along the glass plate. If the points of the legs are moved through the same distance along vertical lines, then the point of the index rod will also describe a vertical line. On the other hand, if the respiratory amplitudes of the points compared are not the same, the point of the index rod will shift to the one side or the other. Therefore, such a lateral shift signifies an existing difference between the tensions of the two sides of the abdominal wall. In reality the various parts of the abdominal wall move during respiration laterally as well as vertically. However,

these lateral displacements are small compared to the vertical ones, and thus, as a simple geometrical construction shows, only the vertical differences need be considered.

The examination can be made most conveniently when the breathing is purely abdominal and the movements of the abdominal wall are not too small. Most of the subjects may easily be induced to breathe in this manner, particularly so if they are told to breathe with an open mouth. But whatever the type of breathing may be, if the movements of the abdominal wall are large enough, the examination gives clear-cut results. If the intra-abdominal pressure increases, that is, if the abdominal wall moves outward, the index point moves toward the more tense side; if the intra-abdominal pressure decreases, that is, if the abdominal wall moves inward, the shift of the point is to the less tense side; and no sidewise shift signifies the absence of a difference in tension. Since the difference between the respiratory amplitudes changes with various depths of breathing, one should observe the angle between the vertical and the line described by the index point rather than the lateral shift of the index point. Other things being equal, the greater this angle, the greater the difference of tensions of the two sides. A graph of the path of the index point is recorded on the glass plate if it is coated by lampblack. In cases showing differences in tension of the two sides, this curve usually consists of three parts: a lower vertical part, corresponding to deep expiration; a slightly S-shaped part which makes an angle with the vertical, and corresponds to the middle phase of respiration; and an upper vertical part, corresponding to deep inspiration. Experiments made with an unevenly stretched rubber membrane showed that an inanimate structure can behave similarly. If the anterior and posterior abdominal walls are forced apart by a solid organ, the corresponding part of the anterior abdominal wall will not move during respiration. In protrusion of one side of the abdominal wall caused by a gas-filled intestinal loop, the difference in level of the two sides is diminished both in deep inspiration and in deep expiration. Thus the index point will describe a shallow C-shaped curve open to the protruded side. In practical work it is not necessary to record the curve traced by the point of the caliper. It is easy to recognize the shape of the curve and to estimate the angle formed by the middle portion of the curve and the vertical line with sufficient accuracy.

TABLE I

| CASE | DEVIATION    |              |         |
|------|--------------|--------------|---------|
|      | UPPER RECTUS | LOWER RECTUS | LATERAL |
| 1    | -            | + L          | + L     |
| 2    | -            | + L          | ++ L    |
| 3    | -            | ++ L         | + L     |
| 4    | + R          | + L          | + L     |
| 5    | + L          | -            | -       |
| 6    | + R          | + L          | + L     |

+ = Slight deviation (up to 15°).

++ = Marked deviation (between 15° and 30°).

R = to right. L = to left.

Fifty male college students, apparently in good health, were examined by the method described. The subject was placed on a table so that his frontal plane was exactly horizontal. Three pairs of symmetrical points of the ab-

dominal wall were used. One pair between the xyphoid process and the navel, 7 cm. apart (upper rectus points), one pair in the level of the navel, 7 cm. apart (lower rectus points), and a third pair 3 cm. below the navel, 14 cm. apart (lateral points). In 28 cases the point of the caliper moved vertically in all three positions of the caliper, showing that there was no difference between the tensions of the two sides of the abdominal wall. Insignificant deviations in some positions of the caliper (less than  $10^\circ$ ) were observed in 16 cases. The results obtained in the remaining 6 cases are shown by Table I.

Observations were also made in a series of patients having abdominal complaints. Deviations greater than  $15^\circ$  (up to  $60^\circ$ ) were observed in cases of acute appendicitis, cholecystitis, duodenal ulcer, splenic tumor, and also in spastic conditions of the gastrointestinal tract.

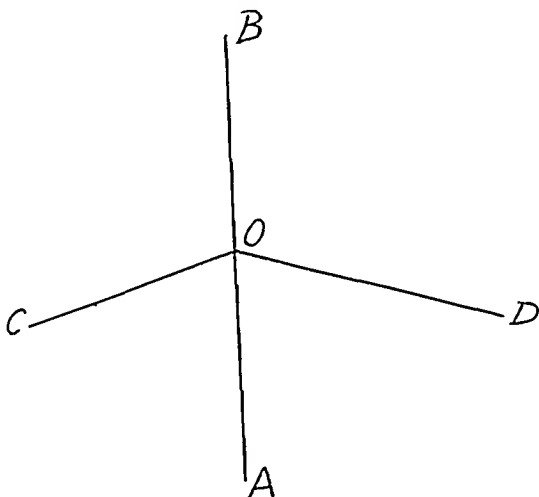


Fig. 2.

These findings were not always in accordance with palpatory findings. This is probably due to the fact that an increase of tension on the side of the pathologic condition may be elicited by palpation itself. Though the observations made in pathologic cases seem to show that the caliper method may have definite clinical value, more extensive observations must be made before the practical importance of this method can be properly established.

#### COMMENT

Obviously, the observed differences between the respiratory amplitudes of symmetrical parts of the abdominal wall are caused by differences between the structures of the two sides of this wall. These differences may be persistent, either acquired or congenital. More interesting from the standpoint of abdominal pathology are the more or less transitory differences caused by unilateral changes in tone\* of the muscles of the abdominal wall. If the tone of one of the rectus muscles increases, the tension on the same side becomes greater and

\*The tone of a muscle is considered to depend upon the percentage of the fibers which are in a state of contraction. If the muscle tone is kept constant, then in a given muscle the tension (which is a purely physical conception) is exactly determined by the length of the muscle.

the respiratory amplitude of every point of this side is smaller than that of the corresponding points of the other side. If the tone of the lateral muscles (transversus and obliqui) of one side increases, an increase of the tension of both sides results. However, the following example shows that the tension of the side having the greater tone will be larger. If two strings AB and CD are tied together at O and stretched in directions, as shown in Fig. 2, the tension along AO will be greater than along BO because O is pulled toward B by COD. Thus a difference of tension is present between two parts of a straight string. In the same way different tensions may be present at various points of the stretched abdominal wall or in various directions about a single point. The difference between the respiratory amplitude of the upper parts of the right and left rectus muscles and the difference between the respiratory amplitude of the lower parts can have opposite signs (Cases 4 and 6, Table I) only if the tensions in various segments of the rectus muscle are different. The fact that a major portion of the muscle fibers originates or ends at the tendinous intersections of this muscle makes it possible that considerable differences in tone be present at the two sides of such an intersection. An increase in tone of a certain part of the rectus muscle causes the interior points of this muscle to move in the direction of this part. In this way the fibers of the lateral muscles are pulled out of position and, therefore, exert an oblique pull on the part of the rectus muscle which increased its tone. A difference between the tensions of parts of the rectus muscle will thus result.

#### SUMMARY

The amplitudes of the respiratory movements of the abdominal wall depend—other things being equal—upon the tension of this wall. By comparing the respiratory amplitudes of symmetrical parts of the abdominal wall while the subject is in an exactly horizontal position, differences between the tensions of the two sides can be demonstrated in an objective manner by using a caliper-like instrument. No significant differences between the tensions of the two sides were observed in 44 out of 50 healthy subjects. In the remaining 6 cases there were marked differences. Differences of a still greater magnitude were observed in various pathologic conditions of the abdominal organs. These observations seem to prove the reality of the palpatory findings of local rigidities of the abdominal wall.

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## RUBBER PERINEUM\*

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FOR the medical student, when he reaches his course in obstetrics, few new experiences offer so much confusion as when he is first called upon to repair a perineal tear or an episiotomy following a delivery. This is so due to the considerable distortion of structures and the abundance of blood in the field.

It is of value for the student to know how to do this relatively simple and common operation before he actually practices upon a patient; the patient profits in his knowing by having a more comfortable and a more perfect repair; the instructor profits by saving of time and disposition at the time of the student's first attempt on a patient; and the student profits by a sense of knowledge and pride in his exactness of technique. Heretofore, to my knowledge, there have been no methods to facilitate the instruction and the practice of this operative procedure other than on the patient.

The device described and illustrated here is of simple construction, is durable enough for about twenty-five practice trials, and may be easily renewed. The student who is quick to learn a new technique can learn to repair an episiotomy in three trials. The method of repair employed will differ with the medical school or with the individual giving the instructions. It is believed that most ordinary methods may be demonstrated accurately and practiced on this model. The main purpose of the device is to teach the student how to lay the sutures in reference to mucosa, and deep and cutaneous tissues, in order to attain a certain degree of perfection before actually repairing a patient.

The essential feature of the device consists of an air foam type of sponge rubber of such a size that an incision simulating an episiotomy may be made across one edge. The consistency is such that the usual curved round surgical needle may be used without tearing the rubber. An oblong piece of bath sponge purchased for ten cents may be cut into two suitable pieces. The sponge is held firmly to a formed base by a system of prongs and spring arms. The base may be made of wood or cast iron with a thin rubber base to prevent slipping on the table top. The spring arms also serve to separate the edges of the incision, exposing what may be termed the "deeper fascial layers"—thus permitting the student to lay deep figure-of-eight or single interrupted sutures. When worn, the sponge may be easily replaced. There is also mounted on the base an upright peg for holding a spool of moderately heavy cotton thread with which the repair is made.

It has been found satisfactory to use a Bartlett Mayo tapered point curved needle. The needle holder may be a discarded one from the operating room or

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any suitable clamp. A pair of seissors or a razor blade completes the essential equipment. With this device one may place sutures simulating deep, mucosal, and subcutaneous stitches with accuracy. By replacing the episiotomy sponge with one in which a simple linear incision has been made, the student may practice ordinary skin closures.

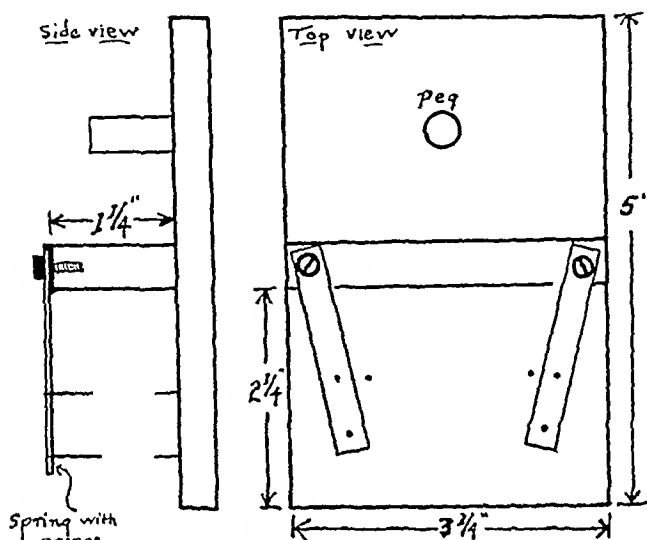


Fig. 1.—Rubber perineum. Scale drawing.

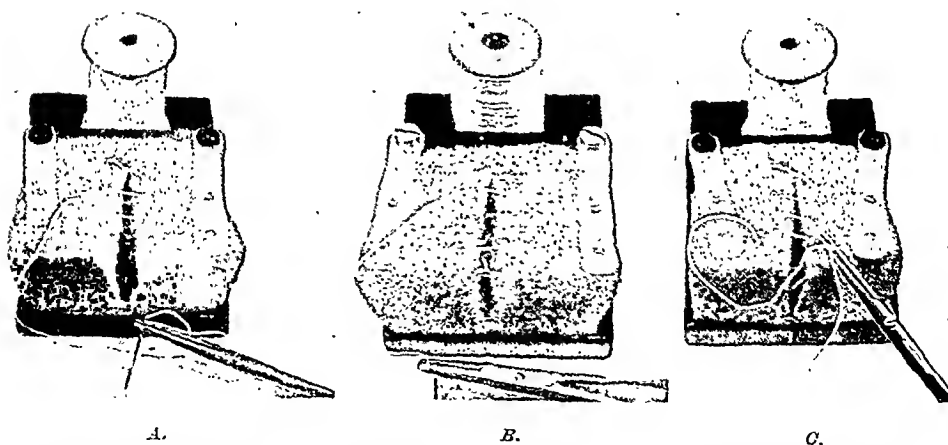


Fig. 2.—Rubber perineum. Photographs illustrating the laying of sutures in the vaginal mucosa, deep structures, and finally the subcutaneous tissues in the repair of a medial episiotomy. Mediolateral episiotomies and tears are very similarly repaired.

Regardless of the amount of material available, this rubber perineum will be of great assistance to the student before he actually attempts a repair on a patient.

Acknowledgment to F. F. Baker, M.D., and Jane Stirling for the photographs.

## A METHOD FOR EXPOSING CULTURES TO CARBON DIOXIDE OR ANAEROBIC ATMOSPHERES IN PETRI DISHES\*

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THE necessity of incubating a few Petri dish cultures under anaerobic conditions or under carbon dioxide tension frequently arises in most bacteriologic laboratories. The need for individual units has been recognized for many years, and two devices of particular merit have been described—the McLeod apparatus<sup>1</sup> and the Spray dish.<sup>2</sup> Both take advantage of the principle of absorption of oxygen by mixtures of alkali and pyrogallie acid. Two compartments are provided in the bottom chamber by means of a built-in barrier, and alkali and pyrogallie acid are placed in the separate compartments. The half of the Petri dish which contains the inoculated agar medium is inverted over the bottom chamber, and the joints are sealed. After sealing, the dish is tipped, effecting a mixture of the solutions.

The Spray dish has been used successfully for growing cultures in carbon dioxide atmospheres produced by mixing sulfuric acid and potassium or sodium bicarbonate by Joyner and Jones.<sup>3</sup>

We have found that the Spray dish technique for anaerobic or carbon dioxide cultivations can be made without the use of specially designed apparatus. The only materials required, in addition to the reagents, are modeling clay and 100 by 15 mm. and 75 by 10 mm. Petri dishes.

Anaerobic cultures are prepared as follows: A rather generous roll of modeling clay is fashioned and placed over the lip of the bottom half of a 100 by 15 mm. Petri dish. A shallow bottom half of a 75 by 10 mm. Petri dish is selected and placed open side up in the larger dish. Four cubic centimeters of a 20 per cent solution of pyrogallie acid solution are run into the bottom of the large dish and 5 c.e. of a 20 per cent sodium hydroxide solution (or 7 c.e. of a 20 per cent sodium carbonate) into the small one. The culture is made in a 100 by 15 mm. Petri dish by pouring inoculated cool agar, or by streaking a hardened agar plate followed by pouring a shallow layer of cooled agar over the surface. The tendency of colonies to coalesce is reduced in such cultures. After the medium has hardened, the half containing the culture is inverted and pressed down firmly into the clay roll. Complete sealing is achieved by flattening the excess remaining on the outside surfaces where the lips of the plates approximate. The reagents are mixed by careful tilting and rotating. The smaller plate slides to the edge of the large one mixing the contents.

An approximate 20 per cent carbon dioxide atmosphere is prepared in the same way, except that 2 c.e. of sulfuric acid solution (1 c.e. of concentrated sul-

\*From the Laboratories of Bacteriology, University of Notre Dame.  
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furic acid in 29 c.c. of distilled water) and 2 c.c. of sterile 1 per cent sodium carbonate solution are used.

Effective anaerobiosis produced by this means has been demonstrated consistently to last for ten days, this being the limit of the observation period. Both methylene blue contained in dextrose beef infusion agar and fresh rabbit blood mixed in agar were used as indicators.

Two per cent agar in thioglycollate medium (Baltimore Biological Laboratories) has been used to advantage in this device. More rapid and perhaps more efficient, anaerobiosis is obtained with this medium under the influence of alkaline pyrogallie acid than occurs with ordinary agar.

#### SUMMARY

A simple method is described by which individual solid media cultures in Petri dishes can be incubated under carbon dioxide tension or under anaerobic conditions.

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### METHOD FOR DETERMINING THE TOXICITY OF ANTISEPTICS AS MEASURED BY THE DESTRUCTION OF HUMAN LEUCOCYTES\*

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A METHOD has been presented previously<sup>1</sup> for determining the primary toxicity of antiseptic solutions, as measured by the loss of the phagocytic function of the leucocyte. By comparing loss of phagocytic activity with the bactericidal action of a compound, it is possible to compute a "toxicity index," which is defined as the greatest dilution of the antiseptic that completely inhibits phagocytosis, divided by the greatest dilution which kills the test organism. Later work<sup>2</sup> indicated that the toxic effect of germicides, when tested by this method on human or guinea pig blood as a tissue, was, in most instances, directed against the humoral elements, in contrast to the cellular elements. However, with mercuric chloride and certain other compounds evidence was presented<sup>3</sup> to show that such solutions, when tested by their effect on hemolytic complement, exhibited definite toxicity but, in contrast to iodine, prevented phagocytosis not by an actual destruction of the humoral or cellular elements involved but by a "blocking" of the effective mechanisms. It was apparent in this type of test that concentrations of antiseptic, which brought about cessation of phagocytosis, were not sufficiently great to cause actual destruction (irreversible loss of function) of the cells.

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It was further apparent from our studies that certain components of the humoral system were sensitive to the toxic action of the majority of antiseptics tested and were thus destroyed by their action, while in those compounds which reacted like mercuric chloride, a "blocking" effect resulted which prevented phagocytosis. This latter effect, however, could be counteracted by dilution of the mixture of plasma and chemical. By testing toxic activity on "naked" cells<sup>2</sup> (blood centrifuged free of plasma), it was shown that greater concentrations of antiseptic were necessary to inactivate such cells than were found necessary to stop their activity when the toxic effect was directed against the humoral system, an essential cog in the phenomenon of phagocytosis.

Through studies of a variety of techniques for determining the effect of antiseptics on the human cell, it soon became apparent that the use of cells free of plasma was neither an efficient nor an appropriate method. Difficulties were encountered when attempts were made to produce a homogeneous cell suspension which would give uniform results when mixed with increasing dilutions of the compound under test. It appeared that relatively viscous cell suspensions failed to contact evenly the solution tested in the amounts used either through purely mechanical improper mixing during rotation or through absence of sufficient amounts of electrolytic solution in the mixture. For example, when mercuric chloride was tested on whole human blood, the toxic dilution was found to be 1:1,300, while on packed "naked" cells mixed with an equal volume of antiseptic dilution a toxic end point of 1:500 was obtained. Although a dilution of 1:600 or 1:700 showed marked phagocytic activity, dilutions of 1:800 nevertheless showed considerably less activity in spite of the lowered concentration of toxic substance. It was apparent that when the mercury salt was present in high concentrations, destruction of all cells was accomplished in spite of improper mixing of cells and test substance due to an excess of the chemical, while at lower more critical concentrations the effect of poor mixing of ingredients becomes obvious.

In the development of the method presented in these studies, consideration was given also to the fact that under the conditions of use in or on the human body antiseptics do not come into contact with naked cells but with serous exudates as well, and this material offers a certain amount of protection to the cell. Furthermore, it seemed particularly desirable to use whole blood, as a tissue, gauging the toxicity of the compound under test by its toxic effect on an individual cell of that tissue, since such a procedure approaches more closely the conditions met when antiseptics are used on other tissues of the body.

Accordingly, in the present investigation a method has been developed to determine the effect on the leucocytes directly, using whole blood as a tissue. Irreversible loss of phagocytic activity is taken as an indication of lethal action on the leucocyte.

#### TECHNIQUE OF TEST

The antiseptic dilutions to be tested are pipetted in 0.1 c.c. amounts to the bottom of test tubes measuring 100 by 13 mm. (Wassermann tubes). To the antiseptic is then added 0.4 c.c. of human blood which has been diluted 1:2 with 0.8 per cent citrated salt solution. (The blood is collected in sufficient 20 per

cent sodium citrate in salt solution to give a final concentration of approximately 0.77 per cent citrate. Usually 20 c.e. of blood are mixed with 0.8 c.e. of 20 per cent citrate.) The mixture of citrated blood and antiseptic is then rotated at 37° C. for ten minutes on a serum-leucocyte mixing machine<sup>1</sup> revolving at 4 r.p.m. As soon as the tubes are removed from the rotator, 2.0 c.e. of 0.8 per cent citrated salt solution are added immediately to each tube to stop further action of the antiseptic. The tubes are shaken vigorously, centrifuged at 2,500 r.p.m. for eight minutes, and the supernatant fluid is removed and discarded. Care should be taken not to disturb the buffy coat of leucocytes usually stratified on the red blood cells when removing the supernatant fluid. The cells which have been in contact with the antiseptic under test for a period of ten minutes are now tested for phagocytic activity by the addition of 0.2 c.e. of antigen and 0.1 c.e. of fresh human plasma. The antigen consists of a standardized suspension of artificially opsonized staphylococci, the method of preparation of which has been described<sup>1</sup> previously. Following addition of antigen and plasma in that order, the mixture is rotated on the serum-leucocyte mixing machine again at 37° C. for twenty minutes. Blood smears are prepared, stained for one minute with 1 c.e. of 0.5 per cent methylene blue in absolute alcohol, following which 1 c.e. of distilled water, buffered at pH 7.2, is added and staining continued for four minutes longer. The degree of phagocytosis is then determined.

TABLE I

| METHOD I   | METHOD II  |
|--|--|
| To show the toxicity of antiseptics as measured by inhibition of mechanism of phagocytosis | To show the toxicity of antiseptics as measured by death of the cell (irreversible loss of function)                             |
| 1. Mixture:<br>0.1 c.e. antiseptic<br>0.2 c.e. antigen<br>0.2 c.e. human blood             | 1. Mixture:<br>0.1 c.e. antiseptic<br>0.2 c.e. citrated salt solution<br>0.2 c.e. human blood                                    |
| 2. Rotated on mixing machine for thirty minutes  | 2. Rotated on mixing machine for ten minutes   |
| 3. Smears prepared and stained with methylene blue for five minutes                        | 3. Two cubic centimeters of citrated salt solution added and mixture centrifuged at 2,500 r.p.m. for eight minutes               |
| 4. Degree of phagocytosis determined   | 4. Supernatant fluid removed and activity of cells tested by the addition of:<br>0.2 c.e. antigen<br>0.1 c.e. fresh human plasma |
|  | 5. Rotated on mixing machine for twenty minutes  |
|  | 6. Smears prepared and stained with methylene blue for five minutes  |
|  | 7. Degree of phagocytosis determined   |

As the concentration of antiseptic is increased, the amount of phagocytosis decreases until a concentration is reached at which no phagocytosis is observed. The degree of phagocytic activity is determined, and complete inhibition of phagocytosis is taken as an end point. The differences between the original method,<sup>1</sup> which measured inhibition of the function of the leucocyte, and the method above, which determines that dilution of antiseptic lethal for the cell,

are shown briefly in Table I. By the use of the two methods described for determining relative toxicity, 26 germicides have been tested. Table II lists the 26 chemical preparations and, wherever possible, the dilution of the active ingredients commonly sold on the market or recommended for use, the dilutions of the active ingredients found toxic by each method, and the corresponding toxic dilutions of the commercial product or recommended concentration.

TABLE II

COMPARISON OF THE TOXICITY OF ANTISEPTICS FOR HUMAN TISSUE AS MEASURED BY INHIBITION OF MECHANISM OF PHAGOCYTOSIS AND DESTRUCTION OF THE CELL

| ANTISEPTIC  | ACTIVE INGREDIENT DILUTION IN COMMERCIAL PRODUCT | DILUTION OF ANTISEPTIC COMPLETELY INHIBITING PHAGOCYTOSIS       |                                |   |                                |
|---|--|---|--------------------------------|---|--------------------------------|
|   |  | WHOLE BLOOD, INHIBITION OF MECHANISM OF PHAGOCYTOSIS (METHOD I) |                                | WHOLE BLOOD, DESTRUCTION OF CELLS (METHOD II) |                                |
|   |  | DILUTION OF ACTIVE INGREDIENT                                   | DILUTION OF COMMERCIAL PRODUCT | DILUTION OF ACTIVE INGREDIENT                 | DILUTION OF COMMERCIAL PRODUCT |
| Acridavine  | 1:1,000  | 1: 2,000  | 1: 2                           | 1:1,200                                       | 1: 1.2                         |
| A (complex organic chlorine compound)             | 1:3,300  | 1: 5,000  | 1: 1.5                         | 1:3,000                                       | Not toxic                      |
| B, Tr. (pyridinium compound)                      | 1: 200   | 1: 5,500  | 1:27.5                         | 1:2,500                                       | 1:12.5                         |
| B, Aq. (pyridinium compound)                      | 1:1,000  | 1: 3,000  | 1: 3                           | 1:2,500                                       | 1: 2.5                         |
| Chloramine-T                                      | 1: 100   | 1: 700  | 1: 7                           | 1: 200  | 1: 2                           |
| *Dodecylamine acetate                             |  | 1:13,000  |                                | 1:8,500                                       |                                |
| †E-605  |  | 1: 900  |                                | 1: 800  |                                |
| †E-607  |  | 1: 1,000  |                                | 1: 900  |                                |
| †E-609  |  | 1: 3,500  |                                | 1:3,000                                       |                                |
| C (resorcinol derivative)                         | 1:1,000  | 1: 5,000  | 1: 5                           | 1:1,700                                       | 1: 1.7                         |
| Iodine tincture                                   | 1: 14.3  | 1: 600  | 1:40                           | 1: 600  | 1:40                           |
| D, Tr. (organic Hg compound + cresol derivatives) | 1: 500   | 1: 7,500  | 1:15                           | 1:3,500                                       | 1: 7                           |
| Mercuric chloride                                 |  | 1: 1,300  |                                | 1:1,300                                       |                                |
| E (organic Hg compound - dye)                     | 1: 50  | 1: 1,700  | 1:34                           | 1: 60   | 1: 1.2                         |
| F, Tr. (organic Hg compound)                      | 1:1,000  | 1:17,000  | 1:17                           | 1:8,500                                       | 1: 8.5                         |
| G (phenyl mercury salt - acetate)                 |  | 1: 1,900  |                                | 1:1,500                                       |                                |
| H (phenyl mercury salt - nitrate)                 | 1:1,500  | 1: 3,000  | 1: 2                           | 1:2,000                                       | 1: 1.33                        |
| I, Tr. (organic Hg - cresol compound)             | 1: 200   | 1: 5,400  | 1:27                           | 1:3,000                                       | 1:15                           |
| Oxyquinoline sulfate                              |  | 1: 700  |                                | 1: 200  |                                |
| Phenol  |  | 1: 400  |                                | 1: 150  |                                |
| Potassium mercuric iodide, aqueous                | 1:5,000  | 1: 1,100  | Not toxic                      | 1:1,100                                       | Not toxic                      |
| Potassium mercuric iodide, 70% alcohol            | 1: 100   | 1: 2,000  | 1:20                           | 1:1,500                                       | 1:15                           |
| Potassium permanganate                            |  | 1: 750  |                                | 1: 200  |                                |
| Sodium ortho-phenylphenate                        |  | 1: 900  |                                | 1: 600  |                                |
| J (cationic detergent)                            | 1:2,000  | 1: 3,000  | 1: 1.5                         | 1:2,500                                       | 1: 1.25                        |
| Zinc sulfocarbolate                               |  | 1: 100  |                                | 1: 20   |                                |

\*This material was obtained from John H. Glynn, M.D. (Armour and Co.) and has been described in the J. Am. Chem. Soc. 63: 1916, 1941; Ibid. 63: 2576, 1941.

†The formulas for these compounds are given in J. Exper. Med. 74: 612, 1941. In a previous publication (J. Immunol. 43: January, 1942) an incorrect interpretation of the chemical make-up of E-605 and E-607 was given.

It will be noted that of the 26 germicides tested, 23 show lower toxicities when toxicity is measured by the direct action on the cells (Method II), while only three show equivalent toxicities by both methods. The marked difference in toxic effect, as measured by the two methods, is brought out particularly with compound "E," which has a toxic end point of 1:1,700 (Table II) when toxicity is measured by the loss of phagocytic function, while a toxic end point of

only 1:60 is obtained when destruction of the leucocyte is taken as an indication of toxic effect. Thus, by the latter method a dilution of only 1:1.2 of the commercial product (2 per cent solution) would represent the toxic dilution of this product, while by the former method a dilution of 1:34 of the commercial product would be toxic. It is of interest that the concentrations of this chemical inhibiting phagocytic function as compared to concentrations which destroy the cell are comparable to those concentrations of this compound which are bacteriostatic and bactericidal.

When destruction of the cell is taken as a measurement of the toxicity of a product such as compound "A," it will be noted that since a 1:3,000 dilution of the active ingredient destroys cells, and the product usually is marketed for use in a 1:3,300 dilution, this product would not be toxic for the cell at the dilution commonly used, although it will be noted that it interferes with the mechanism of phagocytosis at a dilution of 1:5,000. A similar result was obtained with an aqueous solution of potassium mercuric iodide, usually recommended in a 1:5,000 dilution of the active ingredient. As a matter of fact, it will be noted in Table II that besides compound "A" and aqueous potassium mercuric iodide, which are not toxic for the cell in commonly used dilutions, a number of the compounds tested are toxic only at relatively low dilutions of the commonly used strengths when toxicity is measured by direct action on the cell, as exemplified by acriflavine, chloramine-T, and compounds "B," "C," "E," "H," and "J."

#### DISCUSSION

The results obtained by the proposed method, where irreversible loss of function of a human cell is taken as evidence of the toxic activity of antiseptics, indicate that greater concentrations of the compounds tested are required than when reversible loss of function of these same cells is taken as evidence of toxic activity. In the former instance that concentration of antiseptic which destroys the activity of the cell is determined, while in the latter instance the concentration of antiseptic which destroys or interferes with some necessary component of the humoral system is determined.

Where evidence is desired to show the relative toxicity of a group of antiseptics either method may be useful. The method utilized depends upon whether interference with phagocytosis or destruction of tissue cells is the important consideration. In either case the compounds would all be tested under the same set of conditions.

The use of human blood as a tissue and the leucocyte as the test cell seem especially appropriate for measuring toxicity under practical conditions. Human blood is probably the most readily accessible tissue. It is uniform, constant in its pH, reasonably strongly buffered, and in the practical application of antiseptics is the most likely of all the specialized tissues to be encountered. It has been apparent to us in our studies that the leucocyte is relatively resistant to the action of toxic agents and compared to other tissue cells it is unique in its independent nature and its ability to survive for reasonably long periods of time though removed from its natural environment. The resistance of the leucocyte to antagonistic agents, including certain detergents, is rather remarkable and

deserves special comment. With certain dilutions of these compounds it was noted in many instances that where the red blood cells were completely hemolyzed and the plasma coagulated, the leucocytes continued to carry on their phagocytic function undiminished when fresh plasma was added to them. When the resistance of the leucocyte is compared to the resistance of most other body cells *in situ*, it is generally accepted that the latter are probably less affected by toxic compounds than the former. On the other hand, because of their interdependence on one another, individual tissue cells undoubtedly have less resistance than leucocytes to the action of toxic compounds. It is undoubtedly true, as well, that many tissue cells in their natural habitat are destroyed by the toxic action of antiseptics, although such destruction is difficult to determine and becomes apparent only when such large numbers of cells die that the structure or continuity of the tissue is broken down. Therefore, the toxic effect of an antiseptic on the leucocyte may be taken as evidence of a similar if not greater effect on other individual tissue cells. Furthermore, the mechanism of phagocytosis is manifestly an important one in protection of the host against invading organisms, and since the leucocyte acts as one of the antibacterial agents of the body, chemical antiseptics should augment rather than reduce the activity of these cells.

The results obtained with tincture of iodine, mercuric chloride, and potassium mercuric iodide, all three of which showed the same toxic end point by both methods reported, deserve some comment. As previously reported<sup>2</sup> all three of these compounds had a lower toxic end point when tested on the naked cell than when tested on whole blood, using inhibition of the mechanism of phagocytosis as an end point. The differences in toxic end points of these compounds appear to be associated with the type of test used to demonstrate cell destruction and are probably caused by inadequate mixing of cells and the chemical under test. In any case, in the method proposed in these studies special care was taken to study microscopically the slides made from dilutions of these compounds which completely inhibited phagocytosis to be assured that at the toxic end point irreversible loss of phagocytosis through destruction of the cell had actually taken place.

The use of tests of the type described, which give accurate and reproducible results, offers a means of interpretation of the relative merits of a group of antiseptics under the conditions of use on or in the human body, since we are using a viable human tissue and estimating toxic effect on the cell upon which nature relies as one of her first lines of defense against invading microorganisms. It is apparent that the use of the method proposed in these studies will result in lower toxic end points than those obtained by the method previously described.<sup>1</sup> However, the former method, which is somewhat easier to perform, is of value in determining relative toxicity of a group of antiseptics designated for a particular purpose, while the method described in these studies may be taken as an indication of absolute toxicity, since actual destruction of a human cell is involved. The greater value in this method rests in its ability to approach more closely conditions of the use of an antiseptic on human tissue. When the actual resistance of human tissue to the action of an antiseptic is compared

with the resistance of pathogenic microorganisms to the same chemical, an evaluation of the efficacy of the antiseptic becomes possible.

#### SUMMARY

A method for determining the toxicity of antiseptics, which utilizes the destruction of leucocytes as an indication of toxic activity using human blood as a tissue, is presented.

Irreversible loss of the function of phagocytosis following a ten-minute contact with the compound tested is taken as an indication of death of the cell.

The toxicity of 26 antiseptics has been determined by the proposed method, and the results have been compared with those obtained by a method previously described.

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## CHEMICAL

### DETERMINATION OF CHOLESTEROL IN WHOLE BLOOD, SERUM, OR PLASMA\*

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DURING recent years many modifications of Bloor's procedure for the determination of blood cholesterol have been described. The majority of these do not differentiate ester from free cholesterol. Since the esterified form gives more color with the Liebermann-Burchard reagents, it is evident that the value for total cholesterol, as determined by these methods, must be too high. Other factors often leading to high results are colored substances which may be present originally in the blood, as in jaundice, or produced during the extraction process. Schoenheimer and Sperry,<sup>1</sup> and Kelsey,<sup>2</sup> avoid these difficulties by saponifying the esters and removing the cholesterol as the digitonide, which is then purified and analyzed. Ireland<sup>3</sup> first removes the interfering colors with silica, then saponifies the esters and extracts the cholesterol with chloroform. These procedures are quite long and do not lend themselves readily to the needs of the blood chemistry laboratory.

Some years ago one of us (J. C. F.) described a method<sup>4</sup> which eliminated interfering colors by use of a synthetic zeolite (Doucil). In this method esters are not saponified before color development, hence the results are necessarily high. In order to overcome this difficulty the method has been modified to obtain the cholesterol in a free state before solution in chloroform and color development. An added advantage is that free cholesterol can be determined on the same extract.

#### EXPERIMENTAL

*Preparation of Extract.*—One cubic centimeter of whole blood, serum, or plasma is added with stirring to approximately 17 c.c. of acetone -95 per cent alcohol (1:1) in a 25 c.c. volumetric flask. The extract is brought to a boil in hot water and set aside to cool. It is then adjusted to the 25 c.c. mark, mixed thoroughly, and filtered through a rapid filter paper. Ten cubic centimeters of the filtrate are measured into a 25 c.c. Erlenmeyer flask, and 0.2 c.c. of 15 per cent potassium hydroxide is added. The solution is warmed in hot water and placed in an incubator at approximately 55° C. for about one and one-half hours. The flask is then removed, and the alkali is neutralized with 0.4 c.c. of hydrochloric acid so adjusted that it will assure complete neutralization. The hydrochloric acid concentration is approximately 4.9 per cent. After neutraliza-

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tion the flask is placed in water at about  $65^{\circ}$  to  $70^{\circ}$  C., and the contents are evaporated to dryness with the aid of a stream of air played upon the surface of the liquid. The flask is removed, allowed to cool, and 0.4 c.c. of distilled water is added. About 1.3 Gm. of neutralized doucil are added and mixed by moderate shaking. Ten cubic centimeters of chloroform are added, and the flask is stoppered immediately with a cork. The mixture is given a whirling motion off and on for five to ten minutes after which it is filtered through a rapid filter paper. In order to prevent concentration of the solution during filtration the funnel should be covered with a watch glass and filtration should be stopped by removal of the filter paper from the funnel as soon as the greater part of the solution has passed through. Five cubic centimeters of the filtrate are pipetted into a test tube standing in a wire rack in a water bath at  $21^{\circ}$  to  $22^{\circ}$  C. Five cubic centimeters of a standard cholesterol solution in chloroform (1 c.c. = 0.08 mg. of cholesterol) are pipetted into another test tube placed in the same bath. Two cubic centimeters of acetic anhydride are added to each. The tubes are mixed thoroughly and replaced in the bath for a time sufficient to enable the contents of the tubes to come to the temperature of the bath. This usually does not require more than five minutes, but no harm is done if they are left for a much longer period. Even a two-hour period does not affect the final color development. Four small drops of concentrated sulfuric acid, approximately 0.1 c.c., are added to each tube from a dropping bottle with a ground glass pipette. The tubes are then removed one at a time, the contents mixed thoroughly, and replaced in the bath. The bath is placed immediately into a dark cupboard for twenty minutes, after which the colors are compared in a colorimeter with an Eastman 71A filter in the eyepiece. The temperature of the bath at the time of comparison should not have risen above  $23^{\circ}$  C.

When free cholesterol is to be determined, 5 c.c. of the filtrate are measured into a 15 c.c. centrifuge tube, the free cholesterol precipitated as the digitonide and determined by the method of Schoenheimer and Sperry.<sup>1</sup> After solution of the cholesterol digitonide in acetic acid, it has been found advisable to control the temperature by using a water bath in the manner already described, except that a temperature of  $25^{\circ}$  C. is used and at least thirty minutes are allowed for color development.

*Recovery of Cholesterol From Standard Solutions.*—In order to show the complete extractability of cholesterol, aliquots of the standard were evaporated to dryness in 25 c.c. Erlenmeyer flasks, the residue was dissolved in 1:1 alcohol-acetone mixture and was carried through exactly the same procedures as those described for the unhydrolyzed and hydrolyzed extracts. Complete recovery was obtained in each case. The presence of water was found necessary for complete extraction. The amount of water used can be varied within fairly large limits; any amount between 0.4 and 1 c.c. inclusive allowed complete recovery of the cholesterol. Amounts outside these limits resulted in incomplete recovery. In the case of hydrolyzed extracts or alkali-treated standards, it was found necessary to neutralize the alkali before extracting with chloroform. We prefer to neutralize it before evaporation of the alcohol-acetone extract, but equally satisfactory results were obtained by neutralization immediately after evaporation.



TABLE I

CHOLESTEROL CONTENT OF UNHYDROLYZED BLOOD SERUM AS DETERMINED WITH AND WITHOUT DOUCIL

| SERUM NO. | CHOLESTEROL PER 100 C.C. OF SERUM |                   | SERUM NO. | CHOLESTEROL PER 100 C.C. OF SERUM |                   |
|-----------|-----------------------------------|-------------------|-----------|-----------------------------------|-------------------|
|           | WITHOUT DOUCIL (MG.)              | WITH DOUCIL (MG.) |           | WITHOUT DOUCIL (MG.)              | WITH DOUCIL (MG.) |
| 1         | 168                               | 168               | 16        | 120                               | 120               |
| 2         | 243                               | 244               | 17        | 148                               | 139               |
| 3         | 154                               | 150               | 18        | 227                               | 222               |
| 4         | 208                               | 208               | 19        | 188                               | 185               |
| 5         | 171                               | 181               | 20        | 405                               | 397               |
| 6         | 160                               | 163               | 21        | 173                               | 173               |
| 7         | 170                               | 172               | 22        | 162                               | 164               |
| 8         | 319                               | 319               | 23        | 398                               | 393               |
| 9         | 289                               | 289               | 24        | 170                               | 169               |
| 10        | 227                               | 227               | 25        | 170                               | 163               |
| 11        | 208                               | 203               | 26        | 211                               | 213               |
| 12        | 232                               | 225               | 27        | 241                               | 240               |
| 13        | 242                               | 244               | 28        | 150                               | 150               |
| 14        | 282                               | 276               | 29        | 147                               | 146               |
| 15        | 185                               | 188               | 30        | 137                               | 137               |

1 to 15 are normal persons.

16 to 30 are nonjaundiced hospital patients.

TABLE II

COMPARISON OF APPARENT CHOLESTEROL CONTENT AS CALCULATED FROM COLOR DEVELOPMENT BEFORE AND AFTER HYDROLYSIS

| SERUM NO. | BEFORE HYDROLYSIS (MG. PER 100 C.C.) | AFTER HYDROLYSIS (MG. PER 100 C.C.) | RATIO B/A | PERCENTAGE FREE* |
|-----------|--------------------------------------|-------------------------------------|-----------|------------------|
| 1         | 241                                  | 194                                 | 1.25      | --               |
| 2         | 276                                  | 226                                 | 1.22      | --               |
| 3         | 180                                  | 136                                 | 1.32      | --               |
| 4         | 220                                  | 179                                 | 1.23      | --               |
| 5         | 225                                  | 177                                 | 1.27      | --               |
| 6         | 203                                  | 160                                 | 1.27      | --               |
| 7         | 129                                  | 107                                 | 1.21      | --               |
| 8         | 195                                  | 160                                 | 1.22      | 25               |
| 9         | 282                                  | 225                                 | 1.25      | 25               |
| 10        | 175                                  | 140                                 | 1.25      | 29               |
| 11        | 270                                  | 204                                 | 1.32      | 25               |
| 12        | 280                                  | 237                                 | 1.18      | 23               |
| 13        | 750                                  | 717                                 | 1.05      | 85               |
| 14        | 173                                  | 150                                 | 1.15      | 82               |
| 15        | 257                                  | 245                                 | 1.05      | 81               |
| 16        | 115                                  | 100                                 | 1.15      | 79               |
| 17        | 112                                  | 101                                 | 1.11      | 73               |
| 18        | 175                                  | 153                                 | 1.14      | 72               |
| 19        | 209                                  | 195                                 | 1.07      | 64               |
| 20        | 165                                  | 152                                 | 1.09      | 56               |
| 21        | 346                                  | 323                                 | 1.07      | 54               |
| 22        | 336                                  | 318                                 | 1.06      | 52               |
| 23        | 177                                  | 162                                 | 1.09      | 51               |
| 24        | 123                                  | 115                                 | 1.07      | 51               |

1 to 12 are normal sera.

13 to 24 are from jaundiced patients with liver disease.

\*Free cholesterol was determined by Schoenheimer and Sperry's method and is expressed as percentage of the value obtained for total cholesterol after hydrolysis.

*Recovery of Cholesterol From Unhydrolyzed Extracts.*—In these experiments 10 c.c. of the alcohol-acetone extract were evaporated to dryness. The apparent cholesterol content was then determined in the manner already described, except that no potassium hydroxide or hydrochloric acid was used. The values obtained for different sera are given in Table I along with those obtained by simply evaporating an aliquot of the alcohol-acetone extract, dissolving the residue in chloroform and developing the color in the usual way. Since no sera containing an abnormal amount of pigment were used, the amount of pigment present was insufficient to interfere with color comparison even in the absence of doucil. It will be seen that the agreement between the two methods is excellent.

*Recovery of Cholesterol From Hydrolyzed Extract.*—That complete recovery of cholesterol was obtained when a standard was put through the procedure outlined for the hydrolyzed extract has already been mentioned. Evidence for the complete extractability from hydrolyzed serum extracts is shown in Table II. In these experiments comparisons have been made of the color developed by the extract before and after hydrolysis. In 12 normal subjects the ratios varied from 1.18 to 1.32, averaging 1.25. Also included in Table II are a number of typical values on pathologic sera from patients with liver damage in whom a marked increase in the percentage of free cholesterol was obtained. It will be seen that the ratio in all patients is definitely below normal. A ratio of 1 would indicate complete absence of ester cholesterol. Since a slight error in either of the determinations affects the ratio materially, variations in ratio similar to these recorded must be expected.

TABLE III

APPARENT CHOLESTEROL VALUE PER 100 C.C. OF JAUNDICED SERUM DETERMINED WITHOUT HYDROLYSIS, WITH AND WITHOUT DOUCIL

| SERUM NO. | APPARENT CHOLESTEROL CONTENT |                   | ICTERIC INDEX (UNITS) |
|-----------|------------------------------|-------------------|-----------------------|
|           | WITHOUT DOUCIL (MG.)         | WITH DOUCIL (MG.) |                       |
| 1         | 261                          | 241               | 160                   |
| 2         | 342                          | 327               | 152                   |
| 3         | 326                          | 301               | 112                   |
| 4         | 139                          | 125               | 68                    |
| 5         | 279                          | 270               | 44                    |
| 6         | 240                          | 232               | 32                    |
| 7         | 138                          | 136               | 24                    |
| 8         | 129                          | 114               | 22                    |
| 9         | 125                          | 112               | 22                    |

*Removal of Interfering Substances by Doucil.*—It is well known that certain colored substances often present in blood, such as bile pigments, or those produced during the extraction, especially when whole blood is used, will cause high results by interfering with colorimetric readings. This is especially true if a suitable filter is not used in the eyepiece. When doucil is used as described, these interfering substances do not dissolve in the chloroform, and consequently do not interfere with the accuracy of the results. Table III presents values obtained for unhydrolyzed extracts on a number of jaundiced sera with and without the use of doucil. It will be seen that the values for sera of high icteric index are distinctly higher in the absence of doucil. In Table IV are shown a

few typical results showing that doucil will remove the brownish color which forms when whole blood is boiled with alcohol-acetone solution. In these cases three samples of whole blood were taken and added to the alcohol-acetone mixture. One flask was brought just to a boil, another was boiled for five minutes, and the third was boiled for twenty minutes. After cooling, all were diluted to volume and mixed thoroughly. Each was then filtered, and the apparent total cholesterol content was determined without hydrolysis, with and without doucil. It will be seen that when doucil was used, the value obtained for cholesterol was not affected even by long boiling of the blood, but values obtained in the absence of doucil increased progressively as the time of boiling was lengthened, with production of greater amounts of colored products.

TABLE IV

APPARENT CHOLESTEROL VALUE OF WHOLE BLOOD EXTRACTS DETERMINED WITHOUT HYDROLYSIS, WITH AND WITHOUT DOUCIL

Values are expressed in milligrams per 100 c.c. of whole blood

| BLOOD<br>NO. | APPARENT CHOLESTEROL VALUE |                |                   |                |                   |                |
|--------------|----------------------------|----------------|-------------------|----------------|-------------------|----------------|
|              | A                          |                | B                 |                | C                 |                |
|              | WITHOUT<br>DOUCIL          | WITH<br>DOUCIL | WITHOUT<br>DOUCIL | WITH<br>DOUCIL | WITHOUT<br>DOUCIL | WITH<br>DOUCIL |
| 1            | 166                        | 161            | 189               | 161            | 181               | 164            |
| 2            | 234                        | 225            | 243               | 215            | 249               | 222            |
| 3            | 128                        | 123            | 138               | 124            | 146               | 124            |

A, Brought just to a boil during the alcohol-acetone extraction.

B, Boiled for five minutes during the alcohol-acetone extraction.

C, Boiled for twenty minutes during the alcohol-acetone extraction.

# SUMMARY

A relatively simple method for the determination of free and total cholesterol is described. Interfering substances, such as bile pigments or colored substances, produced during the extraction process are removed by means of a commercial synthetic zeolite (doucil).

Commercial doucil is alkaline after drying. Neutralized doucil suitable for use may be obtained from A. H. Taylor & Co., Baltimore, Md.

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## THE USE OF A NEW DIGESTIVE MIXTURE FOR PROTEIN DETERMINATIONS\*

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THE best clinical nitrogen determinations, whether they be of a protein or nonprotein nature, are measured by acid digestion, and the resulting ammonia is determined by the Kjeldahl method or is nesslerized by direct addition of Nessler's solution.

Several digestion mixtures are available to the technician for nitrogen determinations, all of which have their advantages and disadvantages. The speed with which a certain sample of material will digest, that is, change from organic to inorganic material, depends upon the concentration of phosphoric acid present. Folin's digestive mixture<sup>1</sup> has a rather high concentration of phosphoric acid in comparison to the sulfuric acid present. This makes a very powerful and rapid digestive mixture. However, such a high concentration of phosphoric acid usually attacks glass and produces cloudiness in the resulting solution unless the digestion is stopped at the proper time. Dupray<sup>2</sup> has reduced the phosphoric acid content of his digestive mixture for nonprotein solutions so as not to produce the above effects. However, by so doing he has also reduced the speed of digestion itself. To overcome this he added an oxidizing agent, perchloric acid. This makes a good mild acid digestive mixture.

Since proteins have such a high nitrogen content, they require a digestive mixture which is very strong. However, it was my intention to make a strong digestive mixture which would not have too high a concentration of phosphoric acid that would attack pyrex glass, and still would digest with some speed. Furthermore, it must produce clear nesslerized solutions.

In all the various acid mixtures tried the phosphoric acid content was kept to a minimum so as to prevent the etching of glass due to overdigestion. Several oxidizing agents were tried to offset the deficiency of phosphoric acid. Some worked very nicely as far as the digestion was concerned, but on later nesslerization gave considerable cloudiness which rendered it impossible for use in this method since the nitrogen is finally measured by direct nesslerization. Finally, a very fine digestive mixture was realized which had the following composition: 54 c.c. of water, 35 c.c. of sulfuric acid concentrated, 10 c.c. of phosphoric acid 85 per cent, and 0.5 c.c. of perchloric acid 20 per cent. This digestive mixture does not attack glass, and on gentle boiling there is no danger of overdigestion. It gives absolutely clear solutions upon the addition of water after partial cooling. The use of digestive mixtures which have high concentrations of phos-

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phoric acid force one to add water after digestion while still hot in order to avoid the formation of insoluble precipitates. It is thus possible to lose nitrogen due to spurting.

The digestive mixture herein described has one disadvantage and this is that following digestion and dilution with water the resulting solution must be ice-cooled before nesslerization since the nesslerized solutions sometimes turn cloudy when they are not previously cooled. The resulting digested solutions are nesslerized and read either on a colorimeter or photometer. The nitrogen values obtained by this procedure were checked by the Kjeldahl method and compared very well.

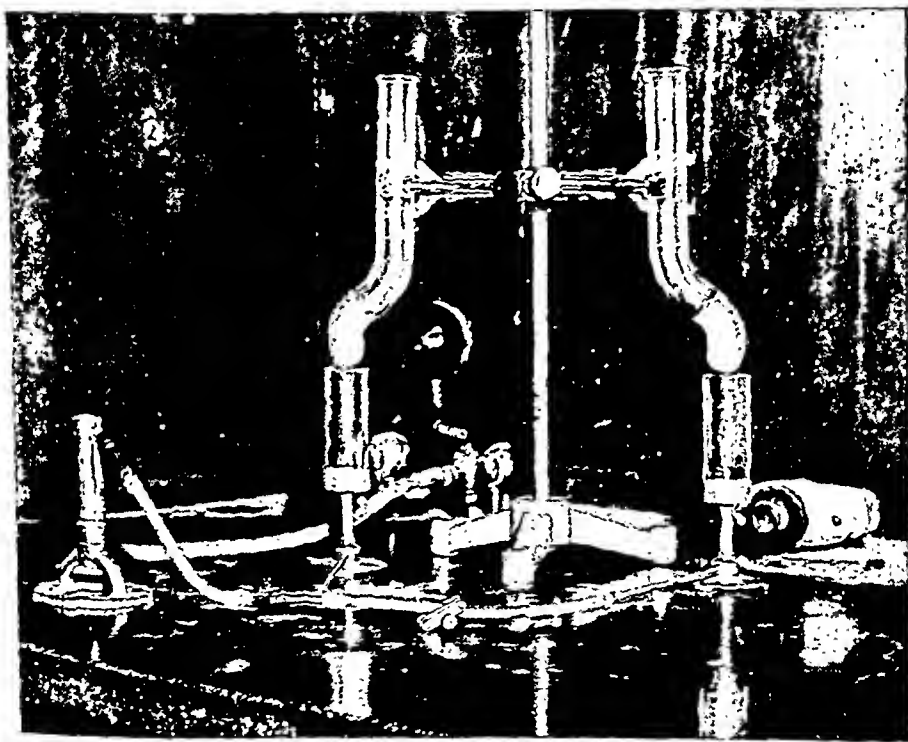


Fig. 1.—Apparatus used for protein digestions.

#### TOTAL PROTEIN

Dilute 1 c.c. serum to 50 c.c. with water and mix well. Take 1 c.c. of diluted serum in a special digestion tube,<sup>\*</sup> previously described.<sup>3</sup> Add 1 c.c. of digestive mixture described above. Digest by boiling gently over a micro chimney burner, as shown in Fig. 1, and cover with a watch glass when charring begins. This takes about fifteen minutes. When it is moderately cool, dilute with distilled water to 35 c.c. Ice cool the latter solution and add 15 c.c. of Nessler's reagent. For comparison in a colorimeter one must use 2 c.c. of standard ammonium sulfate solution, which contains 0.1 mg. nitrogen per cubic centimeter plus 1 c.c. of acid digestive mixture; dilute to 35 c.c., ice cool, and nesslerize. The

<sup>\*</sup>Special digestion tubes may be purchased from Howe and French, 99 Broad Street, Boston.

nitrogen may also be read in a photometer using instead of the standard a blank which is made up of 1 c.c. of acid mixture; dilute to 35 c.c. with water, ice cool, and nesslerize. Calculation:  $S/U \times 0.2 \times \frac{100}{0.02} \times 0.00625 - 0.2 = \text{Gm. of total protein per 100 c.c.}$  (0.2 represents the equivalent amount of protein due to the average nonprotein nitrogen).

#### ALBUMIN

One cubic centimeter of serum is added to 29 c.c. of 22.2 per cent sodium sulfate. Incubate at 37° C. for three or more hours. Filter and discard the first 10 c.c. of filtrate since it will be low in albumin due to absorption by the filter paper. Take 1 c.c. of clear filtrate, add 1 c.c. of digestive mixture described above, and digest in a special digestion tube<sup>3</sup> as described for total protein. Dilute to 35 c.c., ice cool, and nesslerize. The standard is the same as for total protein.

Calculation:  $S/U \times 0.2 \times \frac{100}{0.033} \times 0.00625 - 0.2 = \text{Gm. per 100 c.c. of albumin.}$

Total protein<sup>7</sup> minus albumin gives globulin.

#### SUMMARY

A new digestion mixture is herein described which digests protein and non-protein solutions with the same ease. It does not attack glass, and the digestion does not have to be stopped at the proper moment in order to prevent cloudiness upon the addition of water. A method is described for total protein, albumin, and globulin which is easy, rather rapid, and accurate since there is no loss of material during digestion. This digestion mixture has recently been used for nonprotein nitrogen determinations with great success.

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# QUALITATIVE STUDIES OF THE BILIRUBIN IN BODY FLUIDS

## I. A COMPARISON OF THE DIRECT DIAZO REACTION BY THE PHOTOELECTRIC COLORIMETER, THE THREE TEST TUBE METHOD, AND THE OXIDATION TEST IN SERUM

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IN 1918 Hijmans van den Bergh<sup>1</sup> reported that the addition of diazo reagent to the blood serum of a patient with obstructive jaundice was followed by the prompt appearance of a characteristic color. When the same reagent was added to the serum of a patient with hemolytic jaundice, the characteristic color appeared very slowly, and then only after a considerable lapse of time. When 95 per cent alcohol was added to the serum of a patient with hemolytic jaundice along with the diazo reagent, the characteristic color developed promptly. On the basis of these observations van den Bergh classified the reaction as "direct" when no alcohol was needed to obtain a prompt appearance of color, and as "indirect" when alcohol was required. Since then the literature contains such expressions as "bilirubin direct positive," "direct negative," or "bilirubin indirect positive." These terms, when applied to the qualitative (direct)-diazotization reaction are confusing.<sup>2</sup> The logical terms are direct diazo reaction prompt or delayed. In addition to these two types of reaction, Feigl and Querner,<sup>3</sup> and others<sup>4, 5</sup> described the biphasic reaction, which is a combination of the prompt and delayed reaction.

Since the existing terminology is confusing and since the diagnostic value of the qualitative van den Bergh test is still a matter of dispute, the direct diazo reaction was studied by the photoelectric colorimeter and compared with the three test tube method.<sup>7</sup> The results reported herein will show the comparative value and usefulness of these two methods, and they will also indicate the advantages of using a uniform terminology. In addition, an oxidation test for further differentiation of the various types of bilirubin in blood serum will be described.

### METHODS

**Photoelectric Colorimeter.**—The technique described by Malloy and Evelyn<sup>6</sup> was used. Readings were made at ten, twenty, and forty seconds, and at one, two, three, four, five, ten, and thirty minutes after the diazo reagent had been added to serum. It seemed advisable to obtain colorimetric determinations during the first minute of the reaction, since the qualitative difference between the prompt and delayed type is most striking during this interval. Since Malloy and Evelyn found that the prompt direct diazo reaction attained a maximum of color in thirty minutes, subsequent readings were not considered to be essential.

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**Three Test Tube Method.**—This method originally described by the author<sup>7</sup> in 1923 makes use of the fact that caffeine salts (caffeine sodium benzoate or caffeine sodium salicylate) convert a delayed direct diazo reaction into one which is more or less prompt (Adler and Strauss<sup>8</sup>). Three small test tubes are used. One contains serum and diazo reagent; the second contains serum, diazo reagent, and caffeine salt; the third, serum diluted with water. Using the second and third tubes for purposes of comparison, the nature of the reaction in the first tube can be determined very easily.

TABLE I  
COMPARISON OF QUALITATIVE AND QUANTITATIVE REACTIONS  
CASES WITH PROMPT DIRECT DIAZO REACTION

| DISEASE                              | TOTAL<br>BILIRUBIN<br>(MG. PER<br>100 C.C.) | 1 MIN.<br>DIRECT/30<br>MIN. INDI-<br>RECT<br>(%) | 1 MIN.<br>DIRECT/ 1<br>MIN. INDI-<br>RECT<br>(%) | 1 MIN.<br>DIRECT/30<br>MIN. DIRECT<br>(%) | 30 MIN.<br>DIRECT/30<br>MIN. INDI-<br>RECT<br>(%) |
|--------------------------------------|---|--|--|---|---|
| Catarrhal jaundice                   | 11.8  | 52.3   | 76.6   | 72.4                                      | 72.2  |
| Obstructive jaundice (cancer)        | 6.5   | 68.0   | 90.0   | 64.4                                      | 100.0   |
|                                      | 15.7  | 58.3   | 83.0   | 70.1                                      | 81.2  |
|                                      | 25.6  | 54.5   | 76.9   | 72.1                                      | 75.7  |
|                                      | 18.1  | 46.6   | 69.7   | 60.2                                      | 77.5  |
|                                      | 19.5  | 42.6   | 65.0   | 57.7                                      | 73.8  |
| Obstructive jaundice (stone)         | 32.0  | 42.1   | 61.8   | 67.8                                      | 62.1  |
| Cardiac jaundice                     | 3.2   | 50.0   | 65.4   | 66.8                                      | 70.1  |
| Cirrhosis of the liver with jaundice | 7.8   | 46.9   | 65.9   | 66.6                                      | 70.4  |
| Average                              |   | 51.3   | 72.7   | 66.5                                      | 75.9  |

## RESULTS

*Prompt reactions* were obtained in sera from 6 patients with obstructive jaundice caused by either cancer or stone, one patient with so-called catarrhal jaundice, one patient with liver cirrhosis with jaundice and ascites, and one patient with mild cardiac jaundice. The curves obtained by the photoelectric method showed a steep ascent, a high level in ten to twenty seconds (0.9 to 9 mg. per 100 c.c.), reaching a still higher level within one minute (1.5 to 14 mg. per 100 c.c.). Qualitatively, similar curves were obtained in the patients with obstructive, catarrhal, and cardiac jaundice, even though in the last case the total bilirubin content was only 3.2 mg. per 100 c.c. (Charts 1 and 2). These curves were almost parallel to the curves of the indirect (quantitative) diazo reaction, but, as a rule, they did not reach the same level. There always remained a small amount of bilirubin which reacted with the diazo reagent only after the addition of alcohol. The total bilirubin content of the blood in these cases ranged from 3.25 to 32 mg. per 100 c.c. (see Table I). In order to determine the significance of the various direct curves and their clinical value the figures, as obtained at the beginning of the reaction (one minute) and at the end (thirty minutes), were correlated with each other and with indirect (quantitative) photoelectric curves at one and thirty minutes. Table I shows that in the prompt direct curves, the one minute value reaches on the average 51.30 per cent of the total bilirubin content, 72.73 per cent of the indirect first minute value, and 66.51 per cent of the thirty minute direct readings. When the thirty minute values were



compared with each other, it appears that about 25 per cent of the total bilirubin is usually revealed by the indirect method only.

Further investigation must show whether differences can be determined between the prompt diazo reactions in hepatic and obstructive jaundice. Cantarow, Wirts, and Hollander<sup>9</sup> were unable to find any differences, but Varela and his co-workers,<sup>10</sup> and recently Heilbrun and Hubbard,<sup>11</sup> demonstrated that differences between these types of jaundice exist when they separated the "prompt" and the "delayed" bilirubin quantitatively by the method of chloroform extraction.

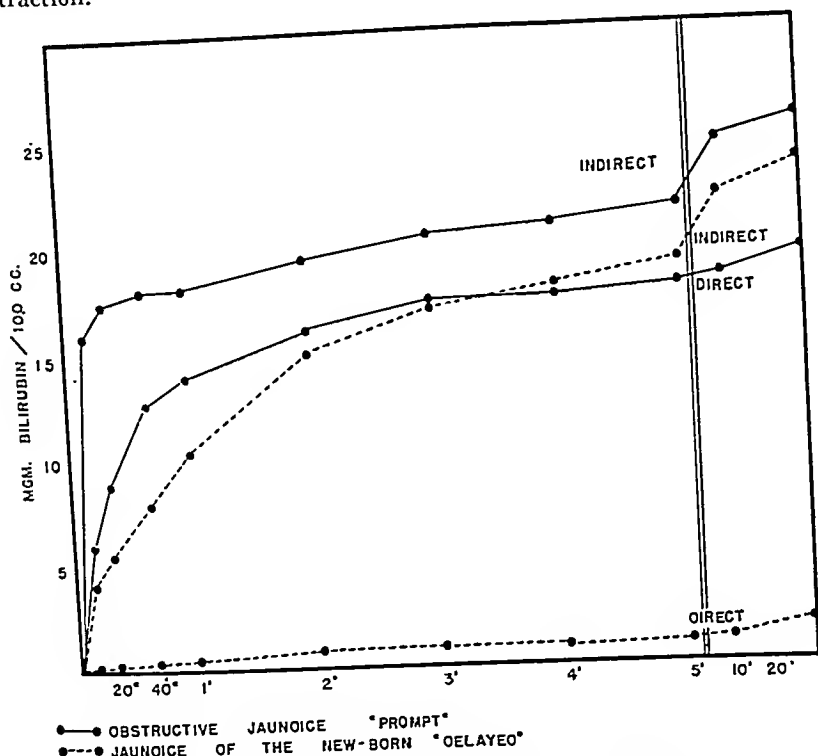


Chart 1.—Comparison of the "prompt direct diazo reaction" in a case of obstructive jaundice with the "delayed direct diazo reaction" in a case of jaundice of the newborn.

In using the three test tube method, a prompt direct diazo reaction was characterized by the appearance of a color immediately after the addition of the diazo reagent. One has the impression that the redness reaches a maximum in one minute (van den Bergh and many other authors). The photoelectric curves, however, reveal that the maximum is reached in about thirty minutes, and Table I shows that the one-minute value of the direct curves is on the average only 66.5 per cent of the thirty-minute direct readings. This fact is due to the accuracy of the photoelectric colorimeter method for detection of the depth of color.

In the three test tube method a new observation was made which may be helpful in the classification of the different types of the direct diazo reaction. In the prompt direct diazo reaction the first orange-red color of the test tube containing the caffeine salts changes to purple in one to two seconds, while in

the tubes containing only serum and reagent the change appears in thirty to ninety seconds. In the delayed and biphasic reactions the appearance of the purple color is much more delayed.

To the *group of delayed direct diazo reaction* belong the serum of a patient with familial nonhemolytic jaundice, 4 patients with jaundice of the newborn, and one patient with healing hepatic jaundice. Furthermore, the following cases of latent jaundice belong in this group: 4 cases of pernicious anemia, 3 specimens of blood from the umbilical cord (placenta blood), one sulfanilamide jaundice (mild case, jaundice of hemolytic origin), one case of possible hemolytic anemia before and after splenectomy. The photoelectric direct curves show a striking contrast to the curves of the prompt group (see Charts 1 and 2). Not only in the first minute but also in the first five minutes a very slow ascent of the curves takes place, and after thirty minutes a low peak is reached. No difference was observed between the curves in the jaundiced cases and those with latent jaundice. But in the cases of jaundice of the newborn and in the case of healing hepatic jaundice the one minute values and the thirty minute values were relatively higher than in the other cases. Apparently the higher total bilirubin content in the jaundice of the newborn and in the healing hepatic jaundice causes the higher readings of the direct curves of these cases.

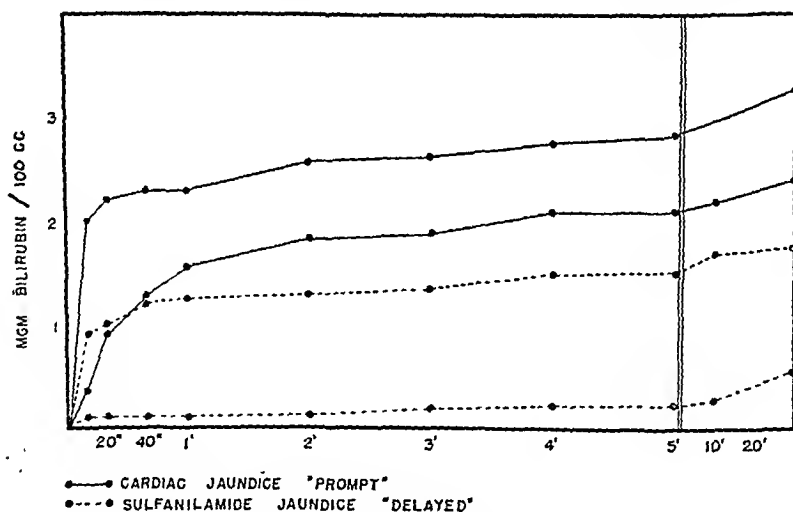


Chart 2.—Comparison of the "prompt direct diazo reaction" in a case of cardiac jaundice with the "delayed direct diazo reaction" in a case of sulfanilamide jaundice.

When the values obtained at various time intervals are correlated with each other, as was done in the cases of the prompt reaction, some striking results were obtained (Table II). In the delayed cases the value at one minute reaches only 9.5 per cent of the total bilirubin in contrast to 51.3 per cent in the prompt group. The other correlations show similar marked differences between the prompt and the delayed direct reaction. The thirty minute direct value reaches only 30.05 per cent of the total bilirubin (75.9 per cent in the prompt cases).

The three test tube method (direct diazo reaction) also shows that the redness in the delayed group develops slowly. The first trace of the orange-red coloring appears five to ninety seconds after the addition of the diazo reagent,

with an average of thirty-nine seconds. Then the color deepens slowly within one to twenty minutes (average five minutes) to a distinct orange red-color. It is of clinical interest that in this group the change to purple color occurs in about ten minutes to two and one-half hours, on the average in about one hour.

In the cases of jaundice of the newborn the development of the color after the addition of the diazo reagent is slower than in the other delayed cases. A distinct orange-red color could be noted only after ten minutes in Case 1 with 6 mg. total bilirubin; after twenty minutes in Case 2 with 7.25 mg.; after twenty-five minutes in Case 3 with 10.7 mg.; and after three hours in Case 4 with a total bilirubin of 23.36 mg. per 100 c.c. In the test tube method the speed with which the redness develops in jaundice of the newborn is apparently in inverse proportion to the total bilirubin content present. The change to purple color, which appears in the tube containing caffeine, does not occur at all in the other tube. Even twenty-four hours, thirty-six hours, and forty-eight hours after the addition of the diazo reagent, a dark orange-red or brownish-red color was present but no purple color. When a few grains of caffeine sodium benzoate were added to an orange-red twenty-four-hour specimen, a change to purple occurred two hours later.

TABLE II  
COMPARISON OF QUALITATIVE AND QUANTITATIVE REACTIONS  
CASES WITH DELAYED DIRECT DIAZO REACTION

| DISEASE                               | TOTAL<br>BILIRUBIN<br>(MG. PER<br>100 C.C.) | 1 MIN.<br>DIRECT/30<br>MIN. INDI-<br>RECT<br>(%) | 1 MIN.<br>DIRECT/1<br>MIN. INDI-<br>RECT<br>(%) | 1 MIN.<br>DIRECT/30<br>MIN. DIRECT<br>(%) | 30 MIN.<br>DIRECT/30<br>MIN. INDI-<br>RECT<br>(%) |
|---------------------------------------|---|--|---|---|---|
| Pernicious anemia                     | 1.3   | 8.2  | 10.0  | 16.2                                      | 51.4  |
|                                       | 1.3   | 21.9   | 33.7  | 41.9                                      | 52.2  |
|                                       | 2.1   | 5.2  | 8.0   | 18.5                                      | 28.2  |
|                                       | 2.4   | 9.3  | 18.5  | 37.7                                      | 24.6  |
| Hemolytic anemia                      | 1.5   | 6.4  | 8.6   | 18.1                                      | 36.1  |
| Hemolytic anemia after<br>splenectomy | 1.6   | 16.5   | 18.3  | 38.8                                      | 42.5  |
| Sulfanilamide jaundice                | 1.8   | 7.2  | 10.5  | 23.7                                      | 30.3  |
| Placental blood                       | 0.9   | 18.4   | 26.4  | 41.9                                      | 46.2  |
| Healing hepatic jaundice              | 1.9   | 8.4  | 9.0   | 24.1                                      | 35.0  |
|                                       | 2.2   | 11.4   | 13.6  | 52.3                                      | 22.8  |
|                                       | 5.2   | 11.3   | 16.7  | 43.7                                      | 26.0  |
|                                       | 7.5   | 10.4   | 16.0  | 56.1                                      | 18.5  |
| Jaundice of newborn                   | 6.0   | 4.3  | 3.1   | 13.2                                      | 33.0  |
|                                       | 10.7  | 7.9  | 12.7  | 38.8                                      | 18.0  |
|                                       | 23.3  | 2.3  | 5.5   | 37.0                                      | 6.8   |
| Average                               |   | 9.5  | 13.4  | 33.4                                      | 30.5  |

This very delayed direct diazo test tube reaction in the cases of jaundice of the newborn seems to be in a certain contrast with the development of color obtained by the direct photoelectric method (average 0.618 mg. per 100 c.c. in one minute, and 1.5 mg. per 100 c.c. in thirty minutes). To explain this discrepancy it may be considered that the deep yellow color of these jaundiced sera may disguise the development of the red coloring in the test tube method.

*Biphasic reactions* were obtained in the sera from the following cases: one of liver cirrhosis, 2 of cholelithiasis with decreasing jaundice, and one of jaundice caused by metastases in the liver. In these cases the direct curves

ranged between the steep ascending curves of the prompt group and the low curves of the delayed group (see Chart 3 and Table III).

In this group the three test tube methods showed two variations which I described in 1921:<sup>5</sup> "biphasic prompt" and "biphasic delayed." In three cases (cholelithiasis with jaundice, liver metastases with jaundice) the reaction was biphasic prompt; immediately after the addition of the diazo reagent a slight red color appeared. The redness deepened more slowly than in the prompt cases but more rapidly than in the delayed cases. The maximum amount of color seemed to be reached after one to two minutes. The change to purple color

TABLE III  
COMPARISON OF QUALITATIVE AND QUANTITATIVE REACTIONS  
CASES WITH BIPHASIC DIRECT DIAZO REACTION

| DISEASE                                 | TOTAL<br>BILIRUBIN<br>(MG. PER<br>100 C.C.) | 1 MIN.<br>DIRECT/30<br>MIN. INDI-<br>RECT<br>(%) | 1 MIN.<br>DIRECT/1<br>MIN. INDI-<br>RECT<br>(%) | 1 MIN.<br>DIRECT/30<br>MIN. DIRECT<br>(%) | 30 MIN.<br>DIRECT/30<br>MIN. INDI-<br>RECT<br>(%) |
|---|---|--|---|---|---|
| Cholelithiasis with jaundice            | 6.7   | 15.9   | 19.4  | 59.7                                      | 26.7  |
| Jaundice caused by cancer<br>metastases | 6.1   | 28.8   | 35.4  | 65.9                                      | 43.7  |
| Cirrhosis of the liver                  | 2.2   | 34.0   | 47.9  | 60.4                                      | 55.4  |
| Cholelithiasis                          | 1.1   | 35.7   | 56.8  | 58.0                                      | 61.6  |
| Average                                 |   | 22.9   | 39.9  | 62.5                                      | 46.8  |

appeared in about one and one-half to four minutes, that is, a little later than in the prompt group. The total bilirubin content in these cases was high (see Table III). In the other cases (2 of liver cirrhosis, 2 of cholelithiasis) the biphasic-delayed direct diazo reaction was present: immediately after the addition of the reagent a very slight red color appeared, but the further development of the color was very slow, similar to the delayed reaction. The maximum color seemed to be reached in two minutes or later. The change to purple in 2 cases with a total bilirubin content of 1.1 mg. and 2.2 mg. per 100 c.c. required twenty and forty-five minutes, respectively.

Besides the differences which can be shown with the direct diazo reaction, van den Bergh<sup>1</sup> reported that the "prompt" bilirubin can be rapidly oxidized to biliverdin, while the "delayed" bilirubin is oxidized slowly. In 1914 Beneke<sup>12</sup> reported that the bilirubin present in blood of jaundice of the newborn, after being treated with formalin, remained yellow, while the bilirubin of cases with obstructive jaundice became green. In 1922 I<sup>5</sup> proposed an easy oxidation test, and as a result of accumulated experience with this test found that it is of value in differentiating the various forms of bilirubin.

#### METHOD

The reagent is made by adding 1 c.c. of 0.5 per cent sodium nitrite solution to 9 c.c. of 1 per cent nitric acid solution. The reagent is prepared freshly each time it is used. The test is performed as follows: 0.2 to 0.3 c.c. of the serum is placed in each of two small test tubes. One tube is diluted with the same amount of water. To the other tube is added 0.2 to 0.3 c.c. of the reagent. Both tubes are held in front of a white paper against the daylight, and the rapidity with which a green color develops is noted during a five to ten minute period.

## RESULTS

In a series of 80 examinations 3 different reactions were observed: (1) "Prompt oxidation test": immediately after the addition of the reagent, a definite green color develops and reaches its maximum intensity in ten to fifteen seconds. The depth of the color depends on the amount of bilirubin present, but it is always green. (2) "Diminished-delayed oxidation test": either a very slight yellow-greenish color develops immediately and changes to a grayish color within two to three minutes, or a rather slight yellow-greenish color develops in thirty seconds to fifteen minutes after the addition of the reagent. (3) "Negative oxidation test": after the addition of the reagent the color of the serum does not change at all or only becomes lighter. Within the next one to three minutes the color changes gradually to a grayish or straw color. No green color is present at any time. Addition of alcohol or of caffeine salts has no influence on the diminished-delayed or on the negative oxidation test.

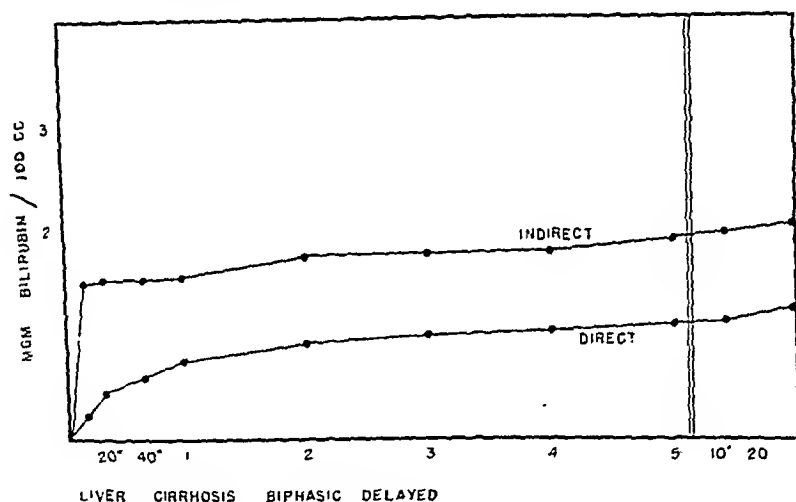


Chart 3.—"Biphasic-delayed direct diazo reaction" in a case of liver cirrhosis.

The *prompt oxidation test* occurred in all cases of obstructive and hepatic jaundice and corresponded to the prompt direct diazo reaction. In one case of mild cardiac jaundice with the low total bilirubin content (3.25 mg.) and in 2 cases with a biphasic-prompt direct diazo reaction (cholelithiasis with 6.7 mg. and cancer metastases in the liver with 6.10 mg. per 100 c.c.) a prompt oxidation test was present. In serum of obstructive jaundice diluted to a bilirubin content of less than 2 mg. per 100 c.c., the prompt oxidation test changed in the majority of the cases to a diminished-delayed reaction.

The *diminished-delayed oxidation test* was observed in cases with delayed or with biphasic-delayed direct diazo reaction (2 cases of liver cirrhosis with total bilirubin of 1.5 mg. and 2.3 mg.; one case of pernicious anemia with a total bilirubin of 1.36 mg.; one case of nonhemolytic familial jaundice with a total bilirubin of 4.06 mg.; one case of sulfanilamide jaundice with a total bilirubin of 1.82 mg.; and one case of healing hepatic jaundice with 5.22 mg. bilirubin per 100 c.c.). The time between the addition of the reagent and the

appearance of the yellow-greenish color did not depend on the amount of the total bilirubin. No case with a prompt or biphasic-prompt direct diazo reaction showed an oxidation test of this type.

The *negative oxidation test* was present in cases with delayed direct diazo-reaction only: pernicious anemia, hemolytic anemia before and after splenectomy, liver cirrhosis, in all specimens of cord blood of the newborn and in the cases of icterus neonatorum. The high total bilirubin content in the cases of jaundice of the newborn (6 mg. to 23.3 mg. per 100 c.c.) shows that also the negative oxidation test was independent of the bilirubin content of the serum. In one exceptional case of cholelithiasis with a total bilirubin content of 1.1 mg. a negative oxidation test was found in the presence of a biphasic-delayed direct diazo reaction.

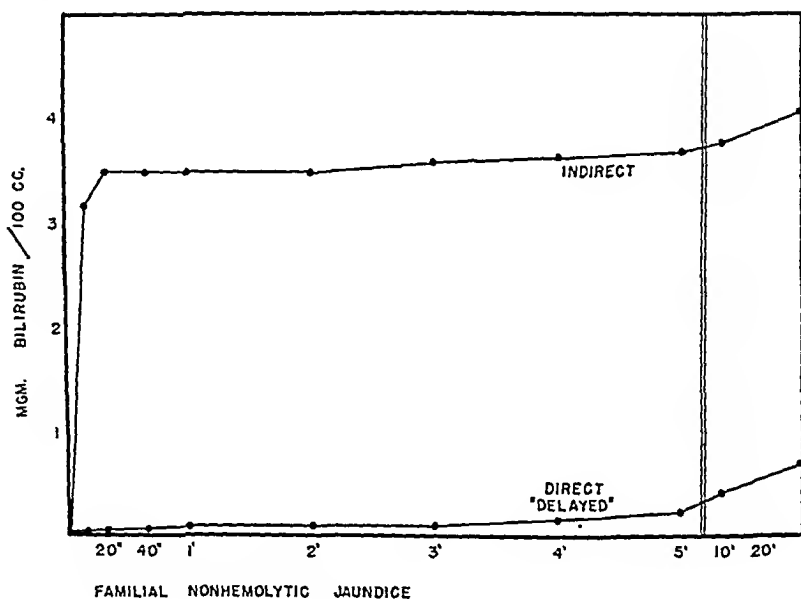


Chart 4.—"Delayed direct diazo reaction" in a case of familial nonhemolytic jaundice.

Photoelectric curves of the different types of this oxidation reaction could not be obtained even though different filters (620, 630, 635) were used. Some examinations with Fouchet's reagent (trichloroacetic acid) and with the oxidation reagent of Malloy and Evelyn<sup>13</sup> (hydrogenium peroxide, concentrated hydrochloric acid, and alcohol) did not show consistent results. Andrewes<sup>14</sup> states that tincture of iodine gives a green color when added to a serum of obstructive jaundice, but an uncertain color when added to a serum of hemolytic jaundice. He obtained similar results with Fouchet's reagent.

#### DISCUSSION

The results presented in this paper show that a real negative direct diazo reaction does not exist. In all cases a positive direct diazo reaction can be obtained by the photoelectric method as well as by the test tube method, but the time necessary for the development of the color and its intensity vary markedly

in the different types of sera. When alcohol and diazo reagent are added to the serum ("indirect" or quantitative reaction), the color appears promptly in every serum. Therefore, it is fair to say that, as far as the qualitative test is concerned, it is not absolutely accurate to differentiate between "direct bilirubin" and "indirect bilirubin" or to speak of "bilirubin direct negative" or "bilirubin indirect positive." It is also confusing that Malloy and Evelyn<sup>6</sup> used the word "promptness" in describing the direct photoelectric curves in cases of hemolytic jaundice which give a definite delayed direct diazo reaction. Bilirubin in the blood serum, when tested by the direct method, is either "prompt," "delayed," or "biphasic."

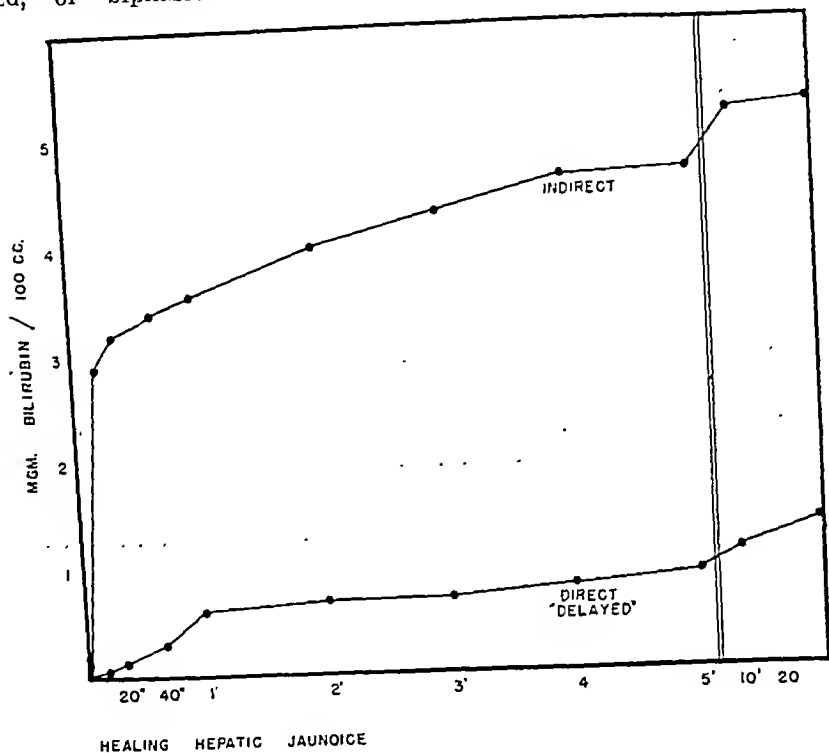


Chart 5.—"Delayed direct diazo reaction" in a case of healing hepatic jaundice.

The qualitative van den Bergh test is believed to be of diagnostic value. When the incline of the photoelectric curves is studied, definite differences appear between the direct prompt, direct delayed, and direct biphasic reaction. By correlating the values at one minute with those obtained after thirty minutes, the difference between the three types of curves is striking. By means of the three test tube method, the prompt, the biphasic prompt, the biphasic delayed, and the delayed types can be distinguished, especially when one takes into consideration the time at which the orange-red color changes to purple. In addition to the direct diazo reaction the oxidation test may be of aid in classifying the different forms of jaundice.

The presence of a prompt or biphasic-prompt direct diazo reaction and a prompt oxidation test exclude an hemolytic jaundice or a jaundice caused by decreased functional ability of the liver cells to excrete bilirubin to the normal

amount (e.g., familial nonhemolytic jaundice) (see Chart 4). These types of the direct diazo reaction and of the oxidation test indicate liver cell damage or an obstruction of the bile ducts. This fact is of particular clinical importance in cases of latent jaundice with a relatively low total bilirubin content. In such cases as cholelithiasis, cardiac failure, liver metastases, and arsphenamine intoxication the direct test tube diazo reaction, if prompt or biphasic prompt, and the oxidation test can reveal the presence of liver cell damage at once. On the other hand, a definite delayed direct diazo reaction and a negative oxidation test (more rarely a diminished-delayed oxidation test) demonstrate the presence of a hemolytic jaundice or jaundice due to functional inability of the liver cells to excrete bilirubin in normal amount or a combination of both. The delayed (sometimes biphasic delayed) diazo reaction and the negative oxidation test exclude hepatic or obstructive jaundice. Only in the early stages and in the healing stages of hepatic or obstructive jaundice a biphasic delayed or even a delayed direct diazo reaction and a diminished-delayed or even negative oxidation test can occur (see Chart 5). In such cases the clinical picture and repeated qualitative examinations of the blood bilirubin in combination with quantitative examinations (icterus index) may help in arriving at the correct diagnosis.

#### SUMMARY

A study of the direct diazo reaction was made with the photoelectric colorimetric method, the three test tube method, and a qualitative oxidation test. The following facts emerged:

1. A prompt, a delayed, and a biphasic type of the direct diazo reaction can be distinguished.
2. The oxidation test can be prompt, negative, or diminished-delayed in various types of jaundice.
3. These methods are of diagnostic value, the most important being the photoelectric colorimetric method.

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## STUDIES ON THE CLINICAL SIGNIFICANCE OF THE SERUM PROTEINS\*

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### I. THE PROTEIN CONTENT OF NORMAL HUMAN VENOUS AND CAPILLARY SERUM AND FACTORS AFFECTING THE ACCURACY OF ITS DETERMINATION

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CHEMICAL changes in the blood are becoming increasingly important in our interpretation and treatment of disease. In order for us either to interpret our clinical data in terms of physiologic or chemical changes or to interpret laboratory data in terms of clinical significance, it is necessary that we study thoroughly the changes of each chemical substance in the body under all circumstances.

To do this we must first of all have laboratory techniques available which are accurate and particularly simple and rapid enough to allow extensive investigation and general use. The methods for hemoglobin, sugar, urine albumin, etc., are simple, rapid, and sufficiently accurate for clinical purposes. They are, therefore, widely done, and their significance is generally appreciated.

In the case of blood protein concentration there has until recently been no test which met all these requirements, and largely for this reason the importance of the blood proteins has been widely underestimated.

It is proposed in this report and in subsequent reports to show the importance of the blood proteins in disease. Herein we shall discuss the normal concentration of blood protein and its two constituents, albumin and globulin, the methods available for determining these concentrations, and details regarding the collection, preparation, and handling of the specimen so as to insure maximum accuracy and greatest ease in obtaining the result.

#### METHODS

The most accurate method for determining the concentration of total protein and its constituents, albumin and globulin, is the Kjeldahl. Unfortunately,

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it is relatively difficult, expensive, and time-consuming. It is, therefore, not generally available nor useful in the sense that the hemoglobin and urinalysis tests are.

There are in existence several colorimetric methods which, while less difficult to use, do not approach the simplicity of usual routine tests, some of which are subject to errors up to 10 per cent.

In 1937 I described a falling-drop technique,<sup>1, 2</sup> which is probably the most rapid and simple method for determining the total serum protein concentration.<sup>3</sup> Duplicate samples of the same specimen analyzed by this method check well within 0.1 Gm. per 100 c.c., and the mean deviation of 107 different specimens of serum when determined by this method and by the macro-Kjeldahl was  $\pm 0.16$  Gm. per 100 c.c. This determination can be done with as little as 0.1 c.c. of blood obtained from finger tip or vein and compares in simplicity with the hemoglobinometer.

It is regrettable that there is no simple test for determining accurately the absolute concentration of albumin alone.<sup>4</sup> There are, however, several simple tests which indicate whether or not the globulin concentration is above normal. To mention only two: the globulin precipitation test<sup>†</sup> and the formol gel test.<sup>‡</sup> If by means of one of these tests it is determined that the globulin concentration is not elevated, then changes in the total protein concentration may be safely attributed to change in the albumin concentration. This will be discussed in detail in subsequent papers on the behavior of albumin and globulin in disease. First, however, let us consider the effect of the manner of collecting, preparing, and handling the specimen on the accuracy of any blood protein determination by any method.

#### DETAILS REGARDING COLLECTION, PREPARATION, AND HANDLING OF SPECIMEN

Rowe<sup>5</sup> and others have found that the protein content of a specimen is increased almost 0.2 Gm. per 100 c.c. for each minute that venous stasis is maintained by a cuff around the arm. This is due to loss of water from the vascular bed. It is, therefore, advisable that blood should be collected within one minute of the application of the tourniquet.

Peters and Van Slyke<sup>6</sup> have reviewed the physiologic factors which may affect the normal concentration of serum proteins. Hyperventilation (when sufficient to produce tetany), vigorous muscular exercise, and stasis of the blood caused by chilling are all capable of increasing the protein concentration. When the body temperature is raised above normal, a lowering of the protein concentration occurs; and when sweating causes sufficient loss of fluid, the opposite obtains. In cases where they are present, these factors need to be kept in mind.

When blood proteins are determined by any of the present colorimetric methods, it is necessary to use blood collected after a twelve-hour fast because

\*The falling-drop principle has been applied for the determination of albumin and globulin, but the necessary precautions are too rigorous for routine use. Since only 27 determinations have been correlated with the Kjeldahl, this method cannot at this time be generally recommended.<sup>4</sup>

†Globulin precipitation test (Naumann<sup>7</sup>): Add 0.1 c.c. of serum to 1.0 c.c. of distilled water in a 10 mm. wide test tube. Saturate with alveolar air and read after fifteen minutes. If definite cloudiness appears, globulin concentration is said to be more than 3.0 Gm. per 100 c.c.

‡Formol-gel (Bing<sup>8</sup>): Add 2 drops of 40 per cent formalin to 1 ml. of serum and read after three hours' standing at room temperature. Globulin concentration is elevated if tube can be inverted without losing contents.

the change in amino acids in blood following food intake affects this method. Such changes in the amino acids do not affect the Kjeldahl or drop methods. In four patients on regular hospital diets, from whom blood was drawn every three hours during the day and hourly after the noonday meal for four hours, there was no change in the serum proteins greater than 0.2 Gm. per 100 c.c. when the determinations were done by the drop method.

The following two experiments done by the falling-drop method suggest that it is advisable that no amount of fluid greater than 500 c.c. be ingested at any one time within four hours of the sampling: (1) Each of 3 persons was given between 600 c.c. and 750 c.c. of water, and their blood proteins were followed at half-hour intervals. All 3 showed decreases in protein concentration which averaged for the 3 a value of 0.15 Gm. per 100 c.c. (2) Each of 3 persons with minor surgical ailments was given 1,500 c.c. of tap water by mouth within five minutes. Their blood changes were similar. The serum protein level fell gradually over the first hour after ingestion of the water to an average of 0.5 Gm. per 100 c.c. less than its original value and returned to the previous level two hours after ingestion. On a subsequent day the same persons were given 1,500 c.c. of normal saline solution in similar manner. The serum proteins fell gradually over a two-hour period to an average of 0.9 Gm. per 100 c.c. less than the original value and were still in 2 cases 0.1 Gm. per 100 c.c. below the original value four hours after the ingestion. Following the ingestion of 500 c.c. of tap water or normal saline, changes in protein concentration are definitely within the experimental error. In view of the foregoing experiments, therefore, it is recommended that no amounts greater than this be ingested within four hours of sampling.

TABLE I

COMPARISON OF THE PROTEIN CONTENT OF SERUM WITH HEPARINIZED AND OXALATED PLASMA

| SUBJECT            | PATIENT 1 | PATIENT 2 | PATIENT 3 |
|--------------------|-----------|-----------|-----------|
| METHOD             | KJELDAHL  | KJELDAHL  | KJELDAHL  |
| Serum              | 4.66      | 6.11      | 7.21      |
| Oxalated plasma    | 4.63      | 6.06      | 7.12      |
| Heparinized plasma | 4.82      | 6.71      | 7.56      |

In many previous investigations and in a number of modern texts no distinction is made between protein values for plasma or serum. The following experiment shows that such a distinction is necessary. Blood was drawn from 3 patients. From blood drawn into a single syringe, serum, oxalated plasma, and heparinized plasma were prepared. The proteins were determined by the macro-Kjeldahl method. The results are shown in Table I. Oxalated plasma may contain the same or lower concentration of protein than serum, even though it would logically be expected to contain about 0.3 Gm. more per 100 c.c. because it contains fibrinogen. Furthermore, the values obtained for oxalated plasma are not as reliable as those for serum, probably because the amount of water withdrawn from the red blood cells varies with the amount of oxalate used as well as with the varying effect that oxalate has on the cells of different persons. The values obtained for oxalated plasma would correspond to those obtained for serum only if the same amount of blood were always mixed together with the same amount of oxalate with an accuracy not usual in the laboratory, and also if it were

true that the cells of every person responded in exactly the same manner to changes in surrounding osmotic pressure. Heparinized plasma may contain as much as 0.65 Gm. per 100 c.c. more protein than oxalated plasma because of a combination of these reasons. In a previous report<sup>2</sup> we found that values obtained for serum protein were definitely more consistent than those for plasma. For these reasons, serum should be preferred to oxalated plasma for use in determining the blood protein concentration.

A series of experiments were done to determine the effect of the age of the specimen on serum protein concentration. These experiments were done by the falling-drop method. A few specimens kept in rubber-stoppered tubes and in an icebox showed increases of over 0.2 Gm. per 100 c.c. when more than twenty-four hours old. Tubes containing the same bloods, but sealed with wax and kept in the icebox, showed no change even after three days. Specimens which were kept in rubber-stoppered tubes at room temperature for twenty-four hours occasionally showed changes as great as 0.7 Gm. per 100 c.c. When cotton-stoppered tubes, such as used in some hospitals, were left at room temperature, increases as high as 1.6 Gm. per 100 c.c. were noted in twenty-four hours. In view of these findings, it would appear safest to do the determinations within twenty-four hours and to keep the specimen in a well-stoppered tube in the icebox.

When a tube of blood is centrifuged without a cork, the concentration of serum protein may rise as much as 0.5 Gm. per 100 c.c. when spun at 3,000 r.p.m. for only fifteen minutes. Although a more detailed analysis of this factor was not attempted, the changes seemed to be directly proportional to the amount of exposed surface relative to the total volume of serum in the tube. When well corked, a tube of blood may be spun at this speed for half an hour without change in concentration.

When the blood was properly stored, there was no difference in the serum protein concentration whether the blood was centrifuged four or twelve hours after its collection or whether or not the serum was separated from the clot before it was stored.

Hemolysis, brought about by shaking a tube of blood vigorously, produced in general an increase of the protein concentration—occasionally of 0.5 Gm. per 100 c.c. Occasionally hemolysis resulted in slight lowering of protein concentration. Apparently the effect depends on the proportion of water and hemoglobin lost from the red blood cells in the procedure. Since it is impossible to judge how great an effect hemolysis will have on an individual specimen, it is considered safer never to use a hemolyzed specimen for this analysis.

In some cases where venous blood is difficult to obtain, it would be very useful to be able to determine the serum protein of blood obtained from the finger tip or heel. This is especially true in pediatrics. Since the amount of blood so obtainable is not adequate for the macro-Kjeldahl technique nor for sufficient accuracy by the colorimetric methods, no comparison of the serum protein concentration of venous and capillary blood has been heretofore reported.

With 0.2 c.c. of blood, which is easily obtainable from the finger tip, about three to five determinations of serum protein can be done by the drop method. It was found most convenient to collect this blood in small test tubes which

measure 35 mm. by 6 mm. and have round bottoms and no lip. A rubber cap fits over the mouth to prevent evaporation. If the finger be placed in warm water for a few minutes prior to being stuck, a free flow is obtained without squeezing tissue fluid into the wound. Capillary blood collected in this manner was compared with venous blood collected from the same arm at the same time. The venous blood was collected without the use of a tourniquet. In tests on 14 adults and 2 children the difference in serum protein concentration between venous and capillary blood was never more than 0.2 Gm. per 100 c.e., and the average difference was 0.07 Gm. per 100 c.e. It is concluded from this experiment that capillary blood is a satisfactory substitute for venous blood and for clinical purposes the results may be considered identical.

#### NORMAL VALUES OF TOTAL SERUM PROTEIN AND OF ALBUMIN AND GLOBULIN

In 1933 Peters and Eisenman<sup>9</sup> reported the value for serum proteins on 52 normal persons. They found that 90 per cent of the values were between 6.3 Gm. and 7.7 Gm. per 100 c.e. This is the largest series reported to date which is based on persons who were known to be normal. Larger series have been reported but not on normal subjects. The purpose of the present report is to add to the data already collected with determinations done by the falling-drop method on 150 ambulatory persons who were normal by examination.

Our analysis was done on the blood of 75 medical students, 25 student nurses, and 50 blood donors. On each student a physical examination, Wassermann test, and urinalysis were done. One student had had a recent appendectomy, one had inactive rheumatic heart disease, and two had orthostatic albuminuria. Their ages ranged from 17 to 35 years. Their physical status was considered good in 52 per cent, robust in 26 per cent, fair in 20 per cent, and asthenic\* in 2 per cent.

The donors were not examined. They had never given blood before and were selected for the most part from men accustomed to hard manual labor in steel and tin plate mills. The results on their blood differ in no respect from that of the others.

The range of serum protein concentration in these 150 persons was 6.1 Gm. to 7.6 Gm. per 100 c.e. The average value was 6.7 Gm. per 100 c.e. The individual male whose serum protein level was 6.1 Gm. per 100 c.e. had had a severe, continuous nosebleed over the previous three days, which finally required cauterization. One month later his serum protein level was 7.0 Gm. per 100 c.e. The female whose protein level was 7.6 Gm. per 100 c.e. was in good health except for being 20 pounds under average weight for her age and height. Another analysis one month later gave a value of 7.5 Gm. per 100 c.e.

Bruckman and associates<sup>10</sup> report an average value of 6.93 Gm. per 100 c.e. for 13 males and 7.6 Gm. per 100 c.e. for 8 females. Peters and Eisenman found no sex difference in their series. The range for the 36 females in this series was 6.3 Gm. to 7.6 Gm. per 100 c.e., with an average of 6.7 Gm. per 100 c.e. The range for the 114 males was 6.1 Gm. to 7.5 Gm. per 100 c.e., with an average of 6.7 Gm. per 100 c.e.

\*For the medical data on these students I am indebted to Dr. Warde B. Allan and Dr. N. B. Herman.

It will be noted that most of the possible deviations from the standard technique used here cause an increase in the serum protein concentration. This may explain why previous investigators have had a few more values between 7.5 Gm. and 8.0 Gm. per 100 c.c. than are found in this report. Many investigations reporting higher values than those reported by Peters and Eisenman and by us have been done on persons who were not normal but who had diseases of various sorts which were assumed at the time not to affect the serum protein concentration. That values above 7.5 Gm. per 100 c.c. are unusual in normal persons is further confirmed by the fact that of 995 determinations of total plasma and serum protein by the macro-Kjeldahl method on 576 patients at the Johns Hopkins Hospital only 24 determinations on 20 patients gave values between 7.5 Gm. and 8.0 Gm. per 100 c.c. Of these 20 patients 15 had diseases which would be expected to cause an increase in the serum protein concentration. This will be discussed in more detail in a subsequent report.

Throughout our work we have accepted the values for normal concentration of serum albumin and globulin to be those given by Peters and Eisenman. They were based upon 50 Kjeldahl determinations on specimens from 34 normal persons. They are for albumin 4.0 Gm. to 5.5 Gm. per 100 c.c. and for globulin 1.4 Gm. to 3.0 Gm. per 100 c.c.

#### SUMMARY

In order to minimize inaccuracies in the determination of blood protein which may arise during collection and handling of the specimen, it is advisable to proceed as follows:

1. Blood should be collected within one minute after the application of the tourniquet.
2. Serum gives more accurate results than oxalated plasma and should, therefore, be preferred.
3. Only nonhemolyzed serum should be used.
4. The blood should be kept in a well-stoppered tube in an icebox, and the analysis should be done within twenty-four hours after collection of the blood.
5. The tube containing the blood sample must also be well stoppered during centrifugation.
6. It is advisable that no amount of fluid greater than 500 c.c. be ingested at any one time within four hours of the sampling. When all the conditions stated are maintained, a difference of protein content of 0.2 Gm. per 100 c.c. from sample to sample in following the course of a disease may be considered quantitatively significant when either the falling drop or the Kjeldahl method is used for all the determinations. This, of course, does not apply when one determination is done by one method and another determination by another method.

Serum from capillary blood obtained from the finger contains for clinical purposes the same amount of protein as venous blood; and the same normal values, therefore, may be applied. Our technique for collecting capillary blood is given.

The range of concentration of serum protein in 150 normal persons was found to be 6.1 Gm. to 7.6 Gm. per 100 c.c., and the average 6.7 Gm. per 100 c.c. Values greater than 7.5 Gm. or less than 6.0 Gm. per 100 c.c. should be con-

sidered abnormal until proved otherwise. There is no difference between the normal values for total serum protein in males and females.

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## THE RELATION BETWEEN SPECIFIC GRAVITY OF BLOOD SERUM AND ITS PROTEIN CONCENTRATION\*

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THE application of the falling-drop method for the determination of specific gravity of blood plasma or serum proposed by Barbour and Hamilton<sup>1</sup> has stimulated the adoption of this technique in many hospitals for the estimation of serum proteins. Because the method for the determination of specific gravity is very precise and accurate, many investigators have been led to the belief that the estimation of serum protein from specific gravity figures by the formula of Moore and Van Slyke<sup>2</sup> is just as reliable. The formula

$$\text{Total Protein} = 343 (\text{sp. gr.} - 1.007),$$

proposed by Moore and Van Slyke, was subsequently confirmed by Weech and his co-workers.<sup>3, 4</sup> These investigators found that the correlation coefficient between specific gravity and total protein had the remarkably high value of 0.994 for serum and 0.991 for plasma. They gave the following equations for the regression line between the two factors:

For Plasma:

$$P = 340.1 (\text{sp. gr.} - 1.00687) \pm 0.103.$$

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For Serum:

$$P = 347.9 \text{ (sp. gr. - 1.00726)} \pm 0.076.$$

However, Zozaya,<sup>5</sup> in a study on the physicochemical characteristics of blood sera, found a correlation coefficient of only  $0.28 \pm 0.062$  between total serum protein and specific gravity. He thought that his failure to obtain better results might have been due to the use of serum rather than plasma. Against this interpretation, however, is the finding of Nugent and Towle<sup>6</sup> that the specific gravity of synthetic solutions of serum albumin and serum globulin was a function of the total concentration of protein regardless of the distributions between the two substances. A recalculation of their data gives the following regression equation:

$$\text{Total Protein} = 366 \text{ (sp. gr. - 1.0067)}.$$

For several years we have been accumulating data on the relationship between the protein content and the specific gravity of blood sera. We have not been able to confirm the findings of Moore and Van Slyke<sup>2</sup> or of Weech and his collaborators<sup>3, 4</sup> that there is a high correlation between specific gravity and serum protein, and that the percentage of protein in a given serum can be predicted with a high degree of accuracy.

#### METHODS

The material for our study has been drawn from three sources: first, from a group of 14 schizophrenic patients who were studied under basal conditions at four different times; second, from a group of 10 normal persons who were studied under basal conditions on two different days; and third, from a group of 12 patients undergoing diathermy therapy who were tested on two different days, before the treatment was begun, at the time that the temperature reached the maximum desired, and finally after the temperature was held at this point for three hours. The specific gravity was determined by the falling-drop method of Barbour,<sup>1</sup> using a solution of potassium chloride of density 1.0343 as the standard of comparison. The total protein was determined by the turbidimetric method of Looney.<sup>7, 8</sup> All determinations were made in duplicate.

The use of the turbidimetric method for the determination of the total protein in our hands has given results which statistically show less variation in duplicate determinations than the standard Kjeldahl method. In a series of 20 sera analyzed for total protein in duplicate by both methods, the following results were obtained: The Howe method gave a mean difference between duplicates of 0.052 mg., with a standard deviation of 0.051 mg. The Looney method gave the values of 0.046 and 0.034 mg. The mean of the differences between means of the duplicates of both series was 0.096 mg., and the standard deviation was 0.069 mg. It is evident, therefore, that there is no significant difference between the methods, and that the variance of the Looney method is somewhat lower than that of the Howe method. In this connection it may be pointed out that only the colorless drops of ghatti gum should be used in the preparation of solutions. If difficulty is experienced in obtaining a good quality of gum, then 2 per cent duponol can be used as a dispersive reagent.



## RESULTS

In Fig. 1 the specific gravity of the sera of the patients on four different days was plotted against the total protein. The values for each person are connected by lines, and the order in which the tests were run is indicated by different symbols.

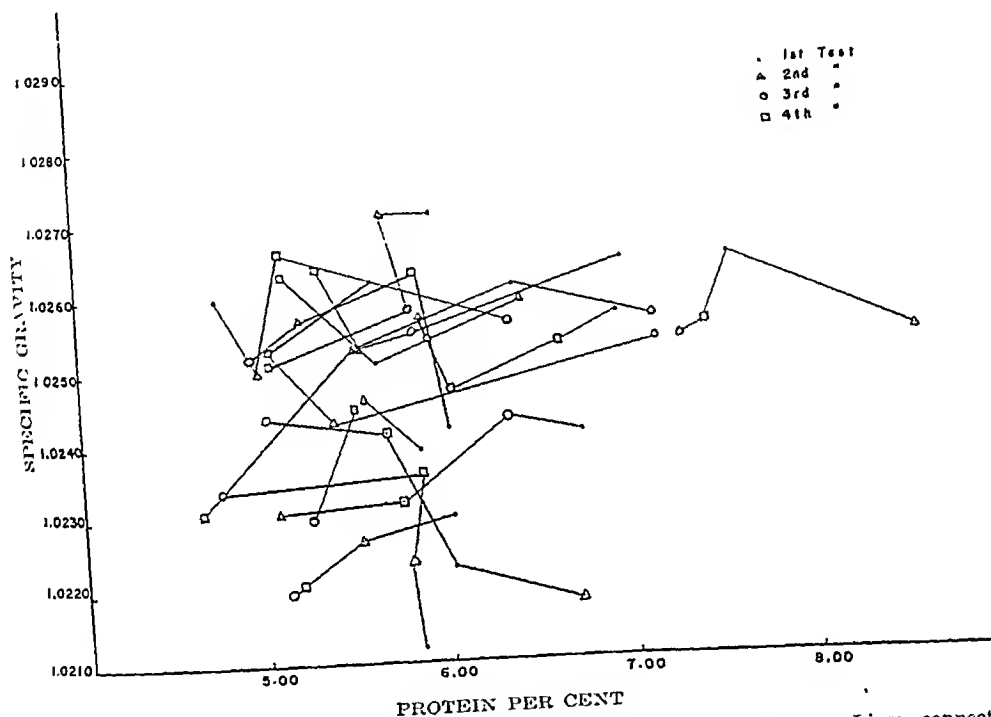


Fig. 1.—Correlation between specific gravity and serum protein in patients. Lines connect readings for individual patients on four different days.

It is evident from an examination of the graph that neither the specific gravity nor the total protein values for any person remain constant. It is also apparent that there is no consistent relationship between the specific gravity and the protein content of the blood serum. The group means for the specific gravity remained practically unchanged from day to day, the values being 1.0248, 1.0246, 1.0247, and 1.0248 for the successive days. The means for the total protein, however, showed considerable variability. The values in grams per 100 ml. of serum were 6.18, 5.87, 5.94, and 5.61. The correlation coefficients for each of these days also showed marked variation, as shown by the following values: 0.22, 0.04, 0.58, and 0.16. The coefficient of correlation for *all* the readings was 0.143.

The means of the total protein for the normal persons were more consistent, giving a value of 6.83 Gm. for the first day and 6.77 Gm. for the second. The specific gravity values gave the same mean 1.0269 for both days. Despite this constancy for the means, the coefficient of correlation was only 0.18. This lack of relationship is clearly shown in Fig. 2.

It was felt that a determination of the relationship between the two variables during diathermy would offer an ideal situation for a conclusive test of the

hypothesis. Marked hemoconcentration takes place during the period of fever despite the administration of copious amounts of water. The protein content of the blood would not be expected to change during the period of the experiment and should furnish a reliable measurement of the amount of fluid loss. If the specific gravity furnishes a reliable index of the total protein content of the blood, then a high degree of correlation should be found in each person tested.

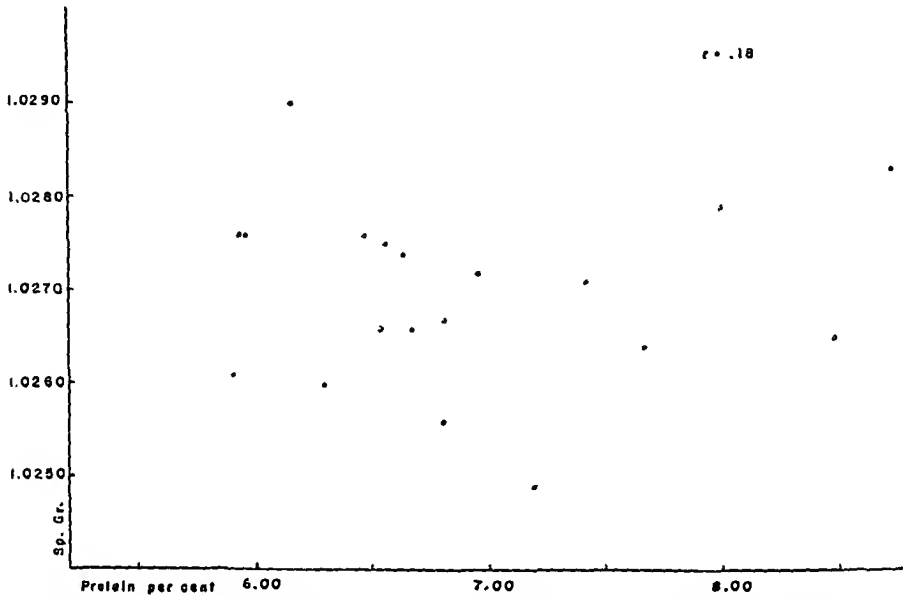


Fig. 2.—Correlation between specific gravity and serum protein in normal subjects.

The results of these experiments are plotted in Fig. 3. It will be noted that in some cases there is such a relationship and the three points can be connected by a line which is straight, or nearly so. In other instances, however, no such relationship occurs, and in one case a fall in specific gravity is associated with an increase in total protein. Moreover, in those cases in which a straight-line relationship was found between the specific gravity and total protein of the serum, the slopes of the lines were not the same. This fact makes the use of any single formula unsuited for the calculation of protein from specific gravity. An inspection of the figure will show that there is a wide spread in the values for specific gravity for the same value of total protein and an even greater spread of total protein for given values of specific gravity. The correlation coefficient for all the values is 0.563 and the regression equation is:

$$\text{Total Protein} = 315 (\text{sp. gr.} - 1.0046).$$

The means for the total protein for the three periods were 6.60, 6.97, and 7.58. The corresponding values for the specific gravity were 1.0258, 1.0267, and 1.0283, and for the hematocrit readings were 40.01, 41.42, and 43.04. The correlation between the serum protein and the hematocrit reading was very low, since the coefficient was only 0.134 and there was no correlation between the specific gravity and the hematocrit reading.

## DISCUSSION

The results of our investigations show that the specific gravity of blood serum is not a simple function of the concentration of protein and that the degree of error in calculating the protein value from the specific gravity in any individual case may be very large. This finding is not unexpected, since the amount of salts and fat in the serum should have considerable influence on the final density of the serum.

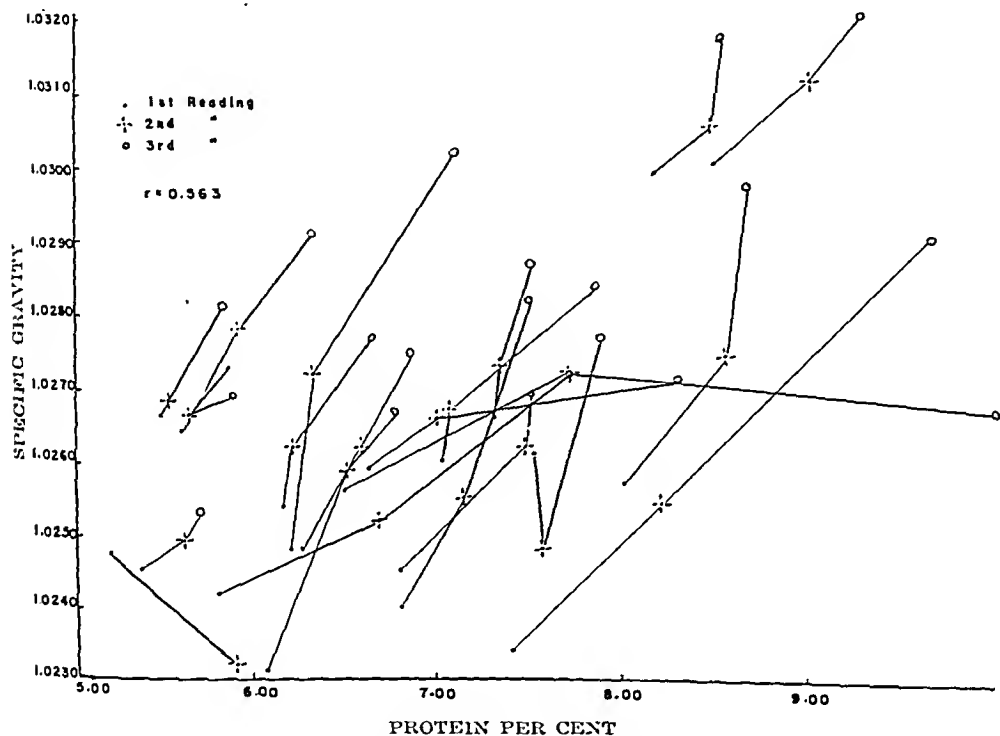


Fig. 3.—Correlation between specific gravity and serum protein during diathermy. Lines connect readings of individual patients.

Our experiments on the changes in protein concentration and specific gravity during the course of diathermic therapy indicate that the concentration of the serum proteins serves as a better indicator of fluid changes in the blood than either specific gravity or hematocrit value. This type of experiment should provide a crucial test of the relationship between density and protein concentration, since the total amount of circulating protein should remain virtually unchanged during the course of the experiment and the concentration should vary directly with the amount of fluid lost from the blood vessels. As a result the study of a smaller number of cases should provide as much information as that of a larger number of unrelated tests on a group of persons selected at random.

It is also evident that, although the amount of change in specific gravity produced by a given change in the concentration of serum protein may be fairly constant in a single person, it is not the same for all persons. The regression equation for a group would give a fair indication of the mean relationship

between the density and the protein concentration, but this would not hold for any single person.

These results are in agreement with the conclusions reached by Zozaya already quoted,<sup>5</sup> and also with the findings given by him in a later paper.<sup>9</sup> He gave figures showing that 12 samples of sera, each having a total protein concentration of 6.59 Gm. per 100 c.c., had specific gravities ranging from 1.0251 to 1.0277. The protein concentration calculated from these specific gravities by the formula of Moore and Van Slyke ranged from 6.21 to 7.10 Gm. per 100 c.c., or using the formula obtained from the data in this paper, from 6.46 to 7.28. In another group of 9 persons, all having a specific gravity of 1.0272 or 1.0273, the total protein values varied from 6.70 to 7.36 Gm. per 100 c.c. The calculated protein for these specific gravities would be 6.93 or 6.96 Gm. per 100 c.c. by Moore's formula and 7.12 or 7.15 Gm. per 100 c.c. by mine. The agreement between the mean protein value of 7.01 Gm. per 100 c.c. and those calculated from the foregoing formulas is good, but the variation from individual readings is rather high.

Similar findings were obtained by Moon and his collaborators<sup>10</sup> in a recent article on shock and hemorrhage. They found that during shock the specific gravity increased while the serum proteins decreased. In hemorrhage, however, both variables were diminished. In these experiments a decrease in the concentration of protein occurred during the period of hemoconcentration following shock, but this loss of protein was not indicated by the specific gravity which increased because of the action of other factors, such as the increased concentration of nonprotein nitrogen and salts except chlorides.

Barbour<sup>11</sup> has adopted the method for the estimation of serum albumin by determining the specific gravity of the supernatant solution from an equal mixture of serum and saturated ammonium sulfate and comparing it with a standard solution of ammonium sulfate. Although the standard deviation for the serum albumin estimated from the specific gravity differed from that obtained by Howe-Kjeldahl method by only 0.32 Gm. per 100 c.c., a study of the graph indicates that for any single case much larger differences may be found. Thus, for a serum albumin value of approximately 3.70 Gm. per 100 c.c., the specific gravity varies from 1.0245 to 1.0268, and for a specific gravity of 1.0245 the serum albumin varies from 2.30 to 3.70 Gm. per 100 c.c. While the method can be used for the determination of the mean value of the serum albumin for any given group within the accuracy indicated by the author, the application to any specific case is fraught with considerable danger since this may involve errors in such instances as great as 50 per cent.

A consideration of the data given in this paper, as well as those obtained from other authors, indicates that the correlation between serum proteins and specific gravity is not sufficiently close to permit accurate estimations of the former variable by a determination of the latter.

#### SUMMARY

The relationship between the specific gravity of serum and its protein concentration was determined repeatedly in a group of schizophrenic patients and also in a group of normal subjects. During diathermy the effect of prolonged

fever on the serum proteins and specific gravity was studied in a third group of parietic patients. The correlation between the two variables was found to be too low to permit accurate estimation of the serum proteins of persons from the specific gravity values.

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# MEDICAL ILLUSTRATION

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## Editorial Comment

In consideration of the fact that physicians often speak during the showing of motion pictures that are neither titled nor recorded in sound, the following article should prove of considerable value as a guide to judging word spacing and timing between sequences. This article should also be of value to the scientific worker who plans to have sound recorded on a previously prepared film. In such a case exact timing is of utmost importance. It is logical to reason that with the development of sound recording devices and the obvious reduction in price that has rapidly taken place in the last few years, the sound motion picture for technical use, as in teaching and reporting at scientific meetings, will eventually replace silent films entirely.

It is worth bringing to the attention of the illustrator and physician the fact that sound can be dubbed in on practically any silent motion picture. Sound projectors and projectionists are usually supplied for the use of speakers at most of the larger meetings. The average sound projector costs about twice as much as a silent projector.

For those persons wishing to study the picture along with the text, prints of the picture may be obtained from the author at a nominal cost.

## TEXT OF THE MOTION PICTURE: A LECTURE ON THE SPIROCHETES\*

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THEODOR ROSEBURY, D.D.S., NEW YORK, N. Y.

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PHOTOMICROGRAPHS OF SOME OF THE PARASITIC AND PATHOGENIC SPIROCHETES AND SPIRILLA AS THEY APPEAR UNDER THE DARK-FIELD MICROSCOPE

(Magnification is uniform throughout, about 160 diameters on the film.)

An approximate scale in microns is given.)

*Note.*—The text is prepared to be read as the film is shown at standard silent speed, 16 frames per second. Numerals in parentheses, e.g., (150), indicate the approximate rate of reading in words per minute. Other directions for reading are also given in italics in parentheses. Titles are given in capitals as section headings.

(*Begin reading on the first dark-field scene*) (150) The spirochetes are distinctive microorganisms, characterized as unicellular spiral filaments with a flexible body, motile without flagella. They are classified among the bacteria as a separate order, but they are sometimes considered intermediate between bacteria and protozoa. (*Wait 5 seconds*) We are going to examine the parasitic spirochetes individually and look into their relationships in disease. Before we do so let us make some general observations on the group as a whole.

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\*From the Department of Bacteriology, College of Physicians and Surgeons, and the School of Dental and Oral Surgery, Columbia University, New York City.

This film was shown at the Forty-Third General Meeting of the Society of American Bacteriologists, Baltimore, December 30, 1941. It was made to be used as a general lecture for medical and dental students. Copies of the film may be obtained at the cost of printing from the negative.

## MORPHOLOGY AND CLASSIFICATION OF SPIROCHETES AND SPIRILLA

(On chart) (120) We recognize five genera of spirochetes, shown here diagrammatically with a group of spirilla for comparison. Let us look at them individually. (Chart builds progressively; time to animation) (150) The genus *Spirochaeta* properly includes only certain large spirochetes. Part of one is shown here. They occur as free-living forms in water and are not pathogenic. They have a spiral axial filament and regularly disposed granules. The genus *Cristispira* is likewise of no medical importance. These spirochetes usually occur as parasites in the alimentary canal of shellfish. They are large forms with a crest or crista, a flexible membrane wound spirally around the spiral organism. They have granules and a chambered structure. The genus *Saprospira*, also not important medically, consists of spirochetes free-living in foraminiferous sand. They resemble *cristispiras*, but have no crest.

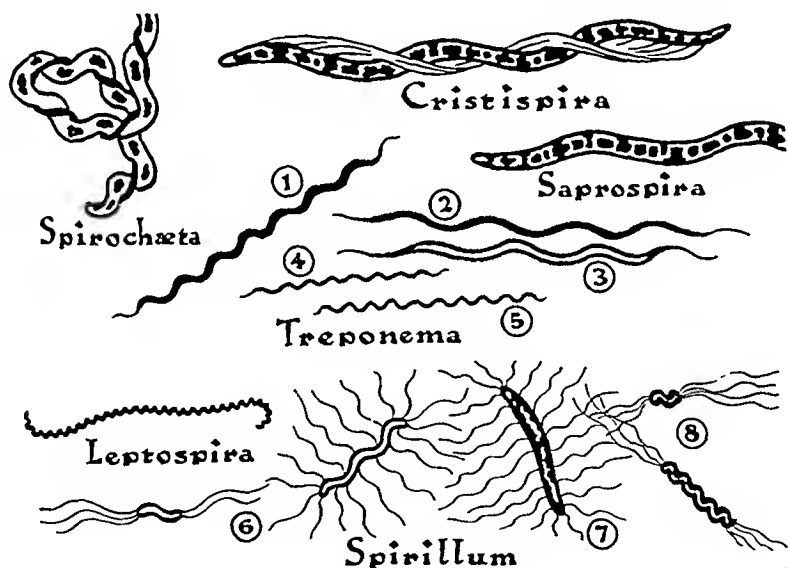


Fig. 1.—Chart of morphology and classification of spirochetes and spirilla. Numbers correspond with those given in the text.

(130) The generic name *Treponema* is used here to include most of the spirochetal species which are parasitic and pathogenic for man and animals. The treponemas usually show no details of internal structure. They are spiral filaments differing in width and in the number and shape of their spirals. These five forms, with their differences exaggerated in the drawings, are all represented in the photomicrographs: (time to animation) 1, the relapsing fever spirochete, intermediate in thickness and winding; 2 and 3, the large spirochetes of mucous membranes, loosely wound, thin, or thick enough to show double contours; 4, the small spirochete of mucous membranes, thin, tightly wound, with shallow spirals; and 5, of which the spirochete of syphilis is the type, thin, tightly wound, with somewhat deeper spirals.

(130) The fifth and last genus of spirochetes is *Leptospira*, which includes both saprophytic and pathogenic species, all of which look somewhat like this.

The leptospiras are much more closely wound than the treponemas, and characteristically one or both ends is bent to form a hook.

(130) The genus *Spirillum* belongs with the true bacteria, not with the spirochetes. The spirilla are flagellated and more or less rigid bacteria. The two forms shown at 6 are typical spirals or portions of a spiral, with flagella peritrichous or polar. Other spirilla, as shown at 7, are curved rods with pointed ends. The rat-bite fever spirillum is 8, short, thick, with rather closely set spirals, and with a tuft of flagella at each end. All these spirilla appear in the photomicrographs.

#### THE DARK-FIELD MICROSCOPE

(On diagram) (120) Dark-field examination at high magnification requires a substage reflecting condenser. This is a bicentric condenser, one of several kinds available, shown in place under a slide with the objective in position. The condenser has two reflecting surfaces, one concave and one convex. The whole condenser-slide-objective system must be made uniform in refractive index; therefore, oil is placed both above and below the slide.

#### PATHWAY OF LIGHT

(150) A narrow pencil of light passes from the mirror to fill the convex reflecting surface, which reflects it so as to fill the concave reflecting surface, and this concentrates the light to form a hollow cone. The cone is focused and centered in the plane of the specimen and in the optical axis. Incident light is stopped by a diaphragm in the objective, and only light reflected off particles in the field reaches the eye. The particles or microorganisms are thus illuminated like dust in a beam of sunlight entering a darkened room: they appear bright against a black background, and look larger or thicker than they really are because we see a halo of light around them.

(On preparation of the slide) (120) In preparing a slide for use under the dark field it is best to use a vaseline seal on the coverslip. Here vaseline is applied from a test tube with a wooden applicator. The seal prevents drying of the film and precludes drifts in the field which result from evaporation at the free edge of the film. (Wait 5 seconds) Both coverslip and slide must be scrupulously clean; bubbles should be avoided in the film since they reflect much more light than do microorganisms. The coverslip is pressed down to make a perfect seal.

(At microscope) (135) A small drop of oil is placed on the cover slip, and a larger drop on the under surface of the slide. A strong light source is used, such as an arc or a concentrated filament lamp. The light is focused through a collecting lens to a bright spot on the plane mirror, and the mirror is adjusted so that a concentrated beam of light fills the convex reflecting surface of the condenser. The objective diaphragm is stopped down, and the condenser is centered and focused so that the apex of the hollow cone of light falls in the optical axis and in the plane of the object. When this is done we see under low magnification (*bright spot appears*) a spot of light like this, which changes symmetrically to a ring of light as the condenser is racked either above or below the focus.



## TREPONEMA RECURRENTIS FROM THE BLOOD OF AN INFECTED RAT

(Clipping tail) (120) A drop of blood from a rat infected with relapsing fever spirochetes is obtained by clipping the tail. The blood must be diluted for dark-field examination in order to separate the cells. The coverslip has previously been prepared with vaseline and a suitable diluting fluid, such as broth.

(On dark field) (150) Here we see red blood cells and *Treponema recurrentis* in a diluted rat blood film under dark field. (Wait 2 seconds) The discovery of these organisms, reported by Obermeier in 1873, brought out not only the first pathogenic spirochete but also one of the first microorganisms of any kind to be associated with disease in man.

(Wait 3 seconds) The motion of all spirochetes can be analyzed into three components: rotation around the long axis, flexion, or bending of the body, and translation or movement from place to place. The relative prominence of these components is a species characteristic, but varies considerably even in a single preparation. These spirochetes show active rotary movements and moderate flexion, but little translation. Others, taken from the same animal, which you will see a little later, show active movements of translation.

(Wait 3 seconds) Relapsing fever in man, as the name implies, is characterized by several sharp paroxysms of fever separated by irregular periods during which the symptoms are absent.

(Progressive movement begins) (150) There are two general types of relapsing fever. One, the so-called European type, is spread from man to man by the body louse, has occurred in severe epidemics, and often accompanies typhus fever. Both diseases are spread by lice, and, as the louse vector suggests, they are associated with poverty and crowding. Louse-borne relapsing fever is caused by *Treponema recurrentis*, a strain of which you see here. (Wait 3 seconds) The other type of relapsing fever is spread by ticks, which parasitize ground squirrels and other wild rodents. This type of the disease in man is sporadic rather than epidemic. It occurs in Africa and in South and Central America. Scattered cases have been reported in several parts of this country. Spirochetes have been recovered on numerous occasions from ticks taken in rodent burrows, especially in the western and southwestern states. The spirochetes of tick-borne relapsing fever are morphologically identical with the louse-borne strain you see here.

(Wait 3 seconds) (150) In relapsing fever in man, spirochetes occur in the blood during the onset phase of each paroxysm and diminish in numbers with its decline. They may be absent or difficult to find during the afebrile intervals. Diagnosis in man is best made by dark-field examination of a diluted blood film such as this, although staining methods can likewise be used on fixed films. The patient's blood should also be injected intraperitoneally into mice or rats, which are then examined for spirochetes in the blood after twenty-four to forty-eight hours. (Elapsed time 12 minutes, 25 seconds)

## THE MOUTH SPIROCHETES AND ASSOCIATED BACTERIA IN EXUDATES FROM FUSO-SPIROCHETAL ANGINA AND GINGIVITIS, AND FROM OTHER MOUTH DISEASES

(On patient) (150) Here we see the method of removing exudate from a case of Vincent's or fusospirochetal gingivitis. To provide enough material for

cultures and animal inoculation, as well as for smears and dark-field examination, (*making suspension*) a heavy suspension is made in 1 c.c. of broth.

(*On dark-field*) (135) Spirochetes similar to those of the mouth may also be found on the genitourinary and intestinal mucous membranes, and in a group of pathologic processes known collectively as the fusospirochetal infections. Several kinds of bacteria always accompany the spirochetes from any of these areas or disease processes. Among these bacteria (*fusiform bacilli appear*) are fusiform bacilli, of which one of several types is shown here; (*motile rod*) various motile bacteria, such as this small organism traveling in a circle, and filaments, as seen at the left; (*vibrio*) also vibrios, sometimes showing one or more polar flagella. Various cocci are always present. (*Spirillum*) Here is a spirillum, a characteristic mouth organism. Note the flagella. (*Motile spirillum*) And here is an actively motile spirillum of a different type.

#### THE MOUTH SPIROCHETES: TREPONEMA VINCENTI AND TREPONEMA BUCCALE

(*On dark field*) (150) These large loosely wound spirochetes of mucous membranes are easily distinguished from the smaller tightly wound forms, particularly when they are in active motion. Notice how these organisms (*spurts*) move forward in spurts, spinning rapidly and stretching their spirals almost taut as they spin, then stopping momentarily, relaxing, and reforming their spirals. This is a characteristic picture.

(*Wait 2 seconds*) (140) The names *Treponema vincenti* and *Treponema buccale* are applied to long loosely wound spirochetes which differ only in thickness. *Treponema vincenti* is the thin form, which appears as a single white line, or, as we say, is single contoured. *Treponema buccale* is thick enough so that light reflected off its opposite surfaces is resolved separately. It, therefore, appears as two white lines separated by a dark line; in other words, it shows double contours. Both spirochetes appear in these preparations, along with smaller spirochetes and various bacteria.

(150) These microorganisms can be demonstrated in small numbers in almost any mouth in which teeth are present, in material taken from the gingival crevice. They are not easy to find on really clean and healthy tissues, and never occur on such tissues profusely. Large quantities are indicative of a pathologic process.

(*On heavy field*) (150) Enormous numbers, like these, may, nevertheless, be found in a wide variety of pathologic conditions, and the number is not an index of severity. The condition may vary from a mild, localized inflammatory or ulcerative process all the way to fatal fusospirochetal gangrene, without any significant difference in the dark-field picture.

(*Double contoured form*) Note the double contours on the spirochete moving vertically. This is *Treponema buccale*.

(*Wait 5 seconds*) (150) Spirochetes found normally or in fusospirochetal lesions, in the anogenital region, have been given distinctive names, although they closely resemble the mouth spirochetes. For example, a genital spirochete called *Treponema refringens* looks like the large spirochetes of the mouth. It is important to distinguish these organisms from the spirochete of syphilis. This is

not hard to do if you remember that these larger nonspecific spirochetes are more loosely wound than the syphilis organism; these spirochetes rotate more actively and move forward much more rapidly when they move. Their spirals are more elastic, being lost and reformed during active rotation. It is worth remembering that *any* spirochete that shows double contours is *not* the spirochete of syphilis. This subject will be discussed more fully later.

(Wait 5 seconds) (150) Another differential feature of these large spirochetes is that they stain fairly well with aniline dyes, whereas the more tightly wound spirochetes, like *Treponema microdentium* and *Treponema pallidum*, do not. The large forms may, therefore, be prominent in stained films; the smaller spirochetes are best seen under dark field, although they can be stained by the Giemsa method or by silver impregnation. Films stained with aniline dyes often are used for the diagnosis of fusospirochetal infections, such as Vincent's angina, the diagnosis being based on the occurrence of these large loosely wound spirochetes and of large granular fusiform bacilli. This procedure may be useful in differential diagnosis, for example, in distinguishing between Vincent's angina and diphtheria; however, its positive value is doubtful because these organisms can be found in large numbers in conditions clinically distinct from Vincent's infection. In lung abscesses and other internal fusospirochetal infections, moreover, these larger organisms may be absent even though smaller spirochetes, small fusiform bacilli, and other bacteria can be demonstrated under the dark field. (End of reel 1; elapsed time 19 minutes)

#### TREPONEMA MICRODENTUM AND OTHER SMALL MOUTH SPIROCHETES

(On dark field) (150) These tightly wound spirochetes of the mouth and other mucous membranes, commonly found along with the larger ones, are interesting for two general reasons. First, the small spirochete called *Treponema microdentium* is morphologically similar to *Treponema pallidum*, so that differentiation of the two forms is important in the microscopic diagnosis of syphilis. Second, the small spirochetes of the mouth seem to be essential members of the fusospirochetal flora, perhaps the most important of the group of microorganisms which appear to be responsible for Vincent's infection. We will consider both points in greater detail as we proceed.

#### MOUTH SPIROCHETES IN EXUDATE FROM INFECTED GUINEA PIGS

(On guinea pig) (150) Material containing the fusospirochetal flora, for instance, the exudate from Vincent's infection, pyorrhea or a lung abscess, when inoculated subcutaneously into the groin of a guinea pig, produces transmissible lesions which may take the form of a large necrotic abscess like this (120) or appear as a fatal diffuse gangrene of the subcutaneous tissues. In either case an abundant exudate, foul and usually fluid, can be aspirated from the lesion with a syringe, as shown here. (Wait 5 seconds) This abscess easily yielded about 5 c.c. of pus; some lesions contain 50 c.c. or even more. (Wait 3 seconds) The pus is frequently greenish, but may be cream colored. (Wait 3 seconds) In this instance it was bloody and dark in color. (On tube) It always has the foul odor characteristic of all fusospirochetal infections.

(On dark field) (120) Examined microscopically, the exudate from a guinea pig lesion usually shows fewer of the large spirochetes, particularly after several passages through guinea pigs. *Treponema microdentium* and other small spirochetes predominate, accompanied by bacteria of fewer varieties than are seen in the original human lesion material. Guinea pig passage eventually eliminates aerobic and gas-forming bacteria and many nonpathogenic anaerobes. The more limited flora that persists seems to represent the more pathogenic types: the smaller spirochetes, smaller nonmotile fusiform bacilli, and gram-negative rods of the *Bacteroides* group, as well as vibrios, streptococci, and other cocci. The whole flora in a stabilized guinea pig strain is anaerobic so that although the exudate contains myriads of living microorganisms, aerobic cultures made from it remain sterile.

(*Pallidum-like spirochete appears*) (150) Here is a form of *Treponema microdentium* which closely resembles *Treponema pallidum*. The two can be distinguished by their motility with some difficulty, as we shall see. When these spirochetes are not actively motile, as in this instance, the distinction becomes extremely difficult, if not impossible.

#### TREPONEMA MICRODENTIUM AND OTHER SMALL SPIROCHETES FROM CULTURES

(On plate) (150) *Treponema microdentium* and the other small spirochetes of the mouth can be cultivated without great difficulty. Here we see a culture of spirochetes in a small Petri plate being sampled with a capillary pipette by removing a portion of the medium containing spirochetes to a cover slip. The spirochetes grow in the depths of the medium, which is a solid nutrient agar containing ascitic fluid or serum ultrafiltrate and mined fresh sterile tissue, inoculated by a special technique and incubated in an anaerobic jar.

(On dark field) (120) *Treponema microdentium* is a small spirochete with regular closely wound coils. Some of the spirochetes seen here are small loosely wound forms, probably distinct from *Treponema microdentium* but as yet of uncertain nature. (Wait 2 seconds) The motility of *Treponema microdentium* is characterized by rapid rotation, often with active movements of translation. When movements of flexion are present, they frequently appear jerky or awkward, thus suggesting that the organism is relatively stiff.

(Wait 5 seconds) (120) These are the spirochetes which appear to be the contributing pathogens in fusospirochetal infections. They predominate in experimental lesions in guinea pigs and in internal fusospirochetal processes in man. Moreover, it has been reported that typical and transmissible fusospirochetal lesions could be produced in guinea pigs with combinations of pure cultures of four species: *Treponema microdentium*, a small fusiform bacillus, a vibrio, and a streptococcus, all anaerobes. Typical lesions could not be produced with any other or any lesser combination of species.

#### TREPONEMA PALLIDUM FROM THE TISSUES OF INFECTED RABBITS

(On rabbit) (160) The testicle of this rabbit, which is infected with *Treponema pallidum*, shows a large chancre. The chancre in rabbits, as in man, is an indurated swelling with surface ulceration that becomes encrusted. Material taken from the indurated tissue under the crust by aspiration with a capillary shows the characteristic spirochetes.

(On dark field) (120) *Treponema pallidum*, sometimes called *Spirochaeta pallida*, varies considerably in length, as do all spirochetes. Otherwise this organism is uniform in morphology, whether taken from the rabbit or from human lesions. Its coils are closely and regularly wound, often a little deeper than those of *Treponema microdentium*. *Treponema pallidum* is delicate; it never shows double contours under dark field. When it is not motile it resembles *Treponema microdentium* so closely that it is difficult, if not impossible, to tell them apart; but the two can be distinguished on the basis of motility. (Wait 2 seconds) *Treponema microdentium*, as we have seen, shows rapid rotation, frequently with active progressive movements. Its movements of flexion, when present at all, are rather awkward and give an impression of stiffness. (Wait 2 seconds) *Treponema pallidum* shows slower, more deliberate movements of rotation. Progressive movement is usually absent. The organism does not dart rapidly across the field. If it moves at all, it usually moves rather slowly and not far. (Wait 2 seconds) The spirochete of syphilis is characterized especially by its flexibility. It appears soft and its movements are, on the whole, graceful. It often looks like a streamer floating gently in the breeze. It has a certain dignity and elegance, befitting the aristocrat of spirochetes. *Treponema microdentium*, on the other hand, is the upstart, nervous and awkward in its movements. *Treponema pallidum* often exhibits a shimmering effect because of the combination of rather slow rotation with soft bending movements.

(Wait 3 seconds) (120) A demonstration of *Treponema pallidum* under the dark field is essential for the diagnosis of primary syphilis, and is also useful for rapid diagnosis in the secondary stage. In either instance, and particularly when the lesion is on or near a mucous membrane, contamination with nonspecific spirochetes should be avoided by expressing or aspirating fluid for dark-field examination from the depths of the lesion after carefully cleaning the surface. When the occurrence of *Treponema microdentium* or similar nonspecific organisms cannot be avoided and positive differentiation cannot be made, it is best to aspirate from a lymph node draining the area of the lesion.

(Sperm appears. Wait 5 seconds) (150) *Treponema pallidum* was not discovered until 1905, when Schaudinn and Hoffmann described it, along with *Treponema refringens*, in preparations made from syphilitic lesions. *Treponema pallidum* cannot be identified by ordinary methods. It cannot be cultivated by any of the methods used to grow bacteria; it does not stain with aniline dyes; and it is so delicate that it can hardly be seen at all in unstained preparations by direct illumination. Schaudinn and Hoffmann, nevertheless, discovered it without the use of dark-field illumination in fresh and in Giemsa-stained preparations.

(Wait 5 seconds) (150) The in vitro cultivation of virulent *Treponema pallidum* remains an unsolved problem. Noguchi and a few others have reported the cultivation of virulent spirochetes; other workers have either failed completely to grow them, or have obtained only avirulent spirochetes in culture which may not have been true pallidum at the beginning. Virulent *Treponema pallidum* is best grown and maintained for study in living tissues, commonly in the testicles of rabbits. In the rabbit testicle it produces a syphiloma or in-

durated swelling which may or may not ulcerate and form a chancre. This lesion usually appears in about three weeks, which is also the average incubation period of the chancre in man. The passage from rabbit to rabbit can be made in indefinite series. This is the Nichols strain of pallidum. It has been passed from rabbit to rabbit for about thirty years. In 1925 it was found pathogenic for man after accidental inoculation.

(Wait 5 seconds) (150) Experimental syphilis has been produced in chimpanzees and in some species of monkeys, as well as in rabbits. In the famous experiments of Metchnikoff and Roux on chimpanzees in 1903, two years before the discovery of the organism, all of twenty-two apes inoculated with human lesion material developed chancres and lymphadenitis, and some had secondary lesions. Most of the animals died of bronchopneumonia and no tertiary lesions developed. (135) Lower monkeys show only primary lesions. Rabbits are easier to infect than apes, and are the animals of choice for in vivo culture and for study of the experimental disease. In addition to the primary lesion they may develop generalized syphilis, with lesions that resemble both secondary and tertiary phenomena in man.

(Wait 5 seconds) (135) Mice, rats, and guinea pigs also can be infected with *Treponema pallidum*, but they usually show no lesions and spirochetes may not be demonstrable in their tissues. Their lymph nodes, spleen, and brain may, nevertheless, remain capable of infecting rabbits.

(On active movement) (120) The spirochetes shown in these preparations are as active as *Treponema pallidum* is ever likely to be. Notice that some of them show progressive movements, but that they move more slowly than *Treponema microdentium*, and much more slowly than the larger loosely wound spirochetes.

#### TREPONEMA PALLIDUM FROM LESIONS IN MAN

(On dark field) (120) These preparations were made from penile chancres. (Wait 2 seconds) Primary lesions in man often yield no more than one or two spirochetes per microscopic field; in fact, it may be necessary to search for the organisms and to make a diagnosis on seeing only one or two typical spirochetes. (Wait 2 seconds) Secondary lesions of the skin also show few spirochetes, as a rule, whereas secondary lesions on or near mucous membranes, mucous patches or condylomas, may show large numbers of pallidum. Skin lesions may, nevertheless, be more suitable for microscopic diagnosis, particularly by the inexperienced worker, because they are much less likely to be contaminated with nonspecific spirochetes. In any case, the surface of the lesion first should be cleaned carefully and fluid for microscopic examination expressed from the depths of the lesion.

(Wait 5 seconds) With a well-adjusted dark field, look carefully for double contours or for active progressive movements. Spirochetes showing either of these are not *Treponema pallidum*.

(Wait 2 seconds) (100) The spirochete of syphilis, as shown here, and this is worth repeating, is delicate, single contoured, and regularly wound with rather deep coils. It is flexible and graceful in its movements, rotates but does not spin rapidly, seldom moves from place to place, and if it does so generally moves rather slowly and not far.

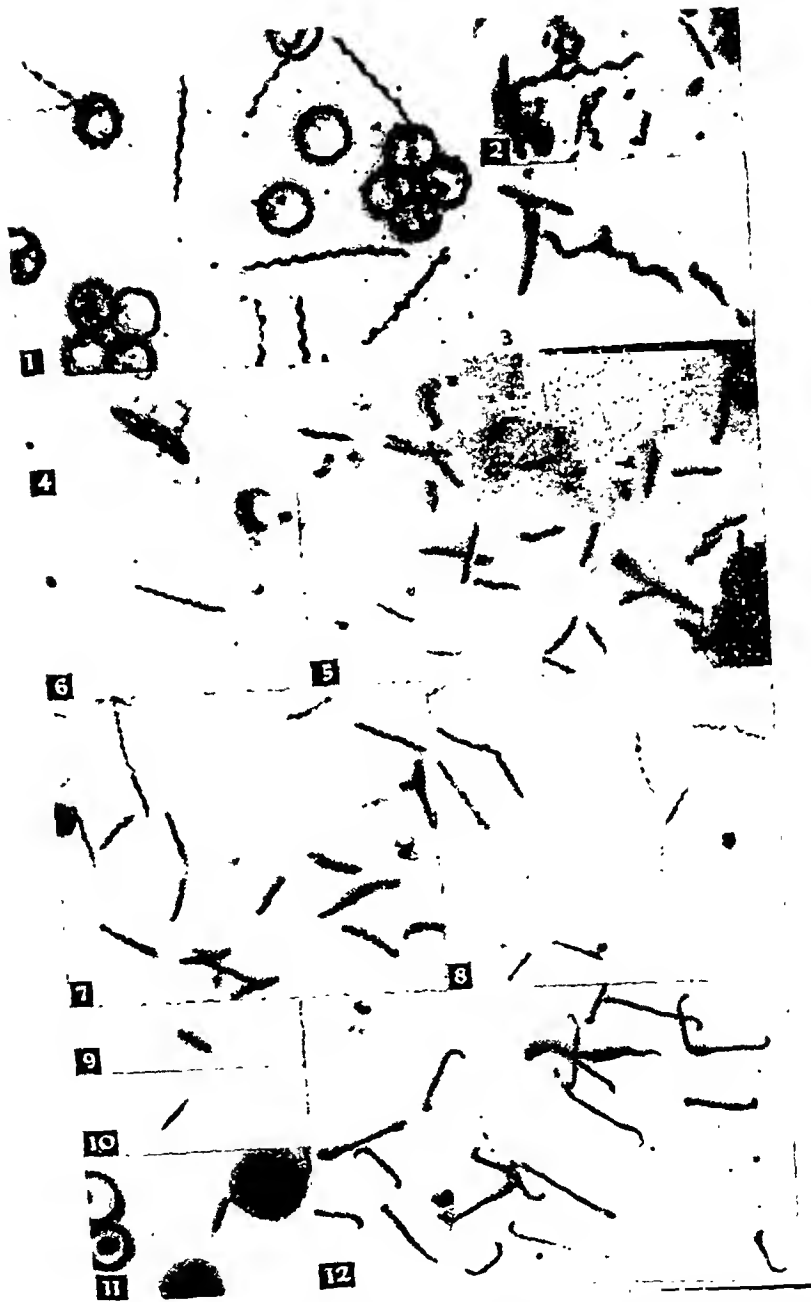


Fig. 2.—All figures are enlargements directly from 16 mm. negative frames. Magnification as reproduced is about 1,100 diameters. 1, *T. recurrentis* (novyi strain) in rat blood; 2 and 3, *T. buccale* and other microorganisms in gingival scrapings; 4, A mouth spirillum (*Sp. sputigenum*!) in gingival scrapings, showing flagella; 5, *T. microdentium*, probably mixed with other small mouth spirochetes, from a culture; 6, *T. pallidum*, from a penile chancre in man; 7 and 8, *T. pallidum* (Nichols strain) from rabbit testicular syphilomas; 9, 10, and 11, *Spirillum minus* in mouse blood and peritoneal washings; 12, *L. canicola* (strain D95) from a culture.

(Wait 5 seconds) (130) While different strains of pallidum are known to vary somewhat in thickness, even under uniform conditions, the organism sometimes appears exceptionally delicate, apparently through an artifact. This may happen if the exudate is kept for some time before microscopic examination, or if only a small amount of exudate is obtained and the film is exceptionally thin (*thin form appears*), particularly if it is made without a vaseline seal. The spirochetes may then look so delicate as to be barely visible, even with the best dark-field equipment. Here you see a preparation of this kind.

#### TREPONEMA CUNICULI FROM AN INFECTED RABBIT

(On dark field) (120) *Treponema cuniculi*, which is shown here, is the causative agent of a naturally occurring venereal disease of rabbits, sometimes called rabbit syphilis, but not to be confused with experimental syphilis produced in rabbits by the inoculation of *Treponema pallidum*. *Treponema cuniculi* and another tissue spirochete, *Treponema pertense*, the causative agent of yaws in man, are both morphologically indistinguishable from the spirochete of human syphilis. Like pallidum, they cannot be cultivated successfully and hence much remains to be learned about their biologic characteristics.

(Wait 3 seconds) (120) Yaws is a nonvenereal disease of man in tropical areas and occurs chiefly in children, with lesions confined mainly to the skin. The lesions of rabbit syphilis usually occur on the skin in the anogenital region, and are much smaller and softer than lesions of true syphilis. The syphilis and yaws spirochetes are immunologically related, but *Treponema cuniculi* is distinct. (End of reel 2; elapsed time 37 minutes, 30 seconds.)

#### LEPTOSPIRA BIFLEXA FROM A CULTURE

(On dark field) (130) This is *Leptospira biflexa*, a nonpathogenic spirochete found in water that contains decomposing organic matter. The several species of *Leptospiras* do not differ in morphology among themselves, but they are easily distinguished from the other spirochetes. They are much more closely wound than any of the treponemas, so that the individual spirals do not always show clearly; and they have the characteristic hook at one or both ends. Their movements are erratic and mercurial, they are the clowns among spirochetes. They rotate rapidly and often dart forward or to and fro; they may show quick lashing movements of flexion, or they may bend into crazy contorted figures.

#### LEPTOSPIRA ICTEROHAEMORRHAGIAE FROM A CULTURE

(On dark field) (120) *Leptospira icterohaemorrhagiae* is the spirochete of Weil's disease or spirochetal jaundice in man. The disease occurs throughout the world and has been reported in several parts of the United States. The organism was first isolated by Inada and Ido in 1915, and independently by Uhlenhuth and Fromme in the same year.

(Wait 5 seconds) (120) Weil's disease, in its more severe form, has a sudden onset with fever which may reach 105° F., and symptoms of acute intoxication. Jaundice may appear after about a week. The mortality may be as high as 50 per cent; but milder cases also occur, more chronic in character, often without jaundice.



(Wait 5 seconds) (120) Whereas all the treponemas appear to be more or less strictly anaerobic and can be cultivated with difficulty, if at all, the leptospiras are aerobic and grow readily in suitable media. They grow at room temperature in the upper centimeter or so of a column of serum or other organic fluid diluted with salt solution and containing a small amount of agar. Actively motile leptospiras like these are easily demonstrated in a loopful of culture removed from the upper portion of the medium and examined under dark field. Like the smaller treponemas, the leptospiras do not stain well with aniline dyes.

(Wait 4 seconds) (120) A characteristic feature of culture leptospiras in active motion is that they rotate so rapidly that their hooked ends look like closed eyelets, as shown by some of these organisms.

#### LEPTOSPIRA ICTERHAEMORRHAGIAE FROM THE LIVER OF AN INFECTED GUINEA PIG

(On dark field) (150) Guinea pigs are exceptionally susceptible to Weil's disease; they usually die about seven to nine days after subcutaneous or intraperitoneal inoculation, and show intense jaundice of the skin and conjunctiva, with leptospiras demonstrable especially in the liver, kidneys, and adrenals.

(Wait 2 seconds) (150) In the diagnosis of Weil's disease a dark-field examination should be made of the blood early in the disease, or of the urine in the later stages, and 2 or 3 c.c. of blood or urine should be inoculated intraperitoneally into guinea pigs. At autopsy, or on about the ninth day after inoculation, dark-field examination of a suspension of the liver should show this picture. Young white mice may be used for inoculation, but older mice are resistant. Species of deer mice have also been used successfully.

(Wait 2 seconds) (150) As we shall see later, certain filaments which appear normally under dark field in blood or tissue suspensions may be mistaken for leptospiras. A true leptospira should show the characteristic hooked ends and at least some degree of rotary motion. These two characteristics justify a diagnosis of leptospira infection even though only a few organisms are found. The movements of flexion are unreliable, as is the apparent demonstration of spirals, since the filament artifacts bend in Brownian movement and may show irregularities that can be mistaken for spirals.

(Wait 2 seconds) (150) The leptospiras are sometimes called the water spirochetes, not only because *Leptospira biflexa* is found in water, but also because contaminated water is directly concerned in the transmission of Weil's disease. The infection has been common in Japan in wet mines in which the miners worked barefoot; elsewhere it has been associated with marshy or muddy soil, or with unsanitary bathing places; and cases have been reported both in Europe and in this country among sewer workers, fish handlers, and others who come into contact with wild rats or rat-infested water. Wild rats are frequently found infected with this organism and excrete leptospiras in their urine. Rats evidently serve as reservoirs of infection and disseminate it by the contamination of water. Transmission to man seems to occur mainly through the skin, the leptospiras being able to penetrate slight abrasions if not the intact skin itself.

TABLE I  
DIFFERENTIAL DATA ON PARASITIC SPIROCHETES AND *Spirillum minus*

| NAME OF ORGANISM  | GENERAL CHARACTERISTICS   | COILS* |             |                      | TYPICAL ACTIVE MOTILITY   |  |  | HABITAT OR SOURCE  | ANIMALS USEFUL IN DIAGNOSIS, ETC.  | NAME OF ORGANISM  |
|---|---|--------|-------------|----------------------|---|--|--|--|--|---|
|   |   | NUMBER | PITCH $\mu$ | OVER-ALL WIDTH $\mu$ | ROTATION  | FLEXION                                | TRANSLATION  |  |  |   |
| <i>T. recurrentis</i> type  | Intermediate in length, thickness, and winding; usually single contoured, may be double under critical illumination | 6-10   | 2.5         | 1.5                  | Moderately active   | Moderate                               | Moderately active; may be absent                               | Blood in relapsing fever during onset of paroxysm; rosette clumps during decline                                 | Mice or rats; blood in frapperi-<br>tonically, spirochetes in animal blood in 24 to 48 hours (stain with aniline dyes) | <i>T. recurrentis</i> type  |
| <i>T. vincenti</i> type<br>( <i>T. refringens</i> )<br><i>T. buccale</i> type | Long, thin; loose shallow coils; single contoured<br>Long, thick; loose shallow coils; double contoured             | 3-6    | 4 or more   | 1-3†                 | Very active; spinning, with heart loss of coils during progression, reformation on stopping | Not marked in fully active forms       | Very active, intermittent spurts in one direction or reversing | Mouth, between gums and teeth; tonsillar crypts; anogenital mucocutaneous areas; fusospirochetal surface lesions | (Stain with aniline dyes)  | <i>T. vincenti</i> type<br>( <i>T. refringens</i> )<br><i>T. buccale</i> type |
| <i>T. microdentum</i> type  | Short, thin; tight shallow coils; single contoured  | 6-12   | 1.2         | 1 or less            | Very active; coils untorted   | May be active, jerky, stiff, or absent | Active, darting; may reverse repeatedly                        | As above; also in internal fusospirochetal lesions (e.g., lung abscess)  | Guinea pigs; exudate subcutaneous in groin; spirochetes in pus aspirated after 4 to 10 days                            | <i>T. microdentum</i> type  |

| <i>T. pallidum</i><br>type          | Medium length,<br>thin to very<br>thin, tight deep<br>coils; always<br>single contoured   | 8-12             | 1.2             | about<br>1   | Rather slow;<br>deliberate                              | Active, soft,<br>graceful                           | Usually ab-<br>sent; never<br>darting | Lesions of primary<br>and secondary<br>syphilis; <i>T. per-<br/>tenuis</i> ; skin le-<br>sions of yaws; <i>T.<br/>cuniculi</i> ; minge-<br>tal lesions of rub-<br>bit syphilis)  | Rabbits (not for <i>T. pallidum</i><br>type)<br>diagnosis; intro-<br>testicular; spiro-<br>chetes in indu-<br>rated tissue in<br>about 3 weeks  |
|-------------------------------------|---|------------------|-----------------|--------------|---|---|---------------------------------------|--|---|
| <i>Leptospiras</i><br>(all species) | Short to long;<br>thin to very<br>thin, single con-<br>toured; very<br>tightly wound;<br>one or both ends<br>hooked (easily<br>cultivated, aro-<br>matic) | 15<br>or<br>more | about<br>0.6    | about<br>0.5 | Very active;<br>spinning;<br>hooks look<br>like eyelets | Marked, with<br>erratic dash-<br>ing move-<br>ments | Active; may<br>be absent              | <i>L. biflexa</i> ; stagnatant water; <i>L. icterohaemorrhagiae</i> ; wild rats and rat-infested water; blood (early); urine (late) in Weil's disease; <i>L. canicola</i> ; dogs | Guinea pigs; blood or urine intraperitoneally; spirochetes in liver, kidneys, adrenals in about 9 days (also cultivation, serologic tests)  |
| <i>Spirillum minus</i>              | Short, thick, but barely double appear closely set under dark field; bipolar flagella   | 2-5              | 1<br>or<br>more | about<br>1   | ?   | Slight or absent                                    | Typical very rapid, darting           | Mouse and rat blood, peritoneal fluid; bite wound and skin papules in rat-bite fever   | Mice intraperitoneally (but spontaneously infection); guinea pigs; blood, excised or excised tissue intraperitoneally or subcutaneously; spirilla in blood or peritoneal washings in 5 to 10 days |

\*These approximate measurements are based on the film. "Pitch" equals the distance between two coils as measured from crest to crest on one side. Length measurements are omitted because wide variation makes them of little differential value. The shortest *T. microdentatum* may be 44, the longest *T. vincentii* about 26 $\mu$ , but some specimens of *T. vincentii* are longer than some *T. pallidum*. Descriptions of motility apply to typical fully active forms; all species lose motility more or less gradually in slide cover slip preparations; dying forms may show erratic movements. †Over-all width decreases during active rotation and progression because of stretching of spirals.

(Wait 2 seconds) (150) Weil's disease, unlike other spirochetal infections, confers lasting immunity. Agglutinins and lysins develop during the disease and persist for many years after recovery.

(Wait 2 seconds) (150) Noguchi, who made many outstanding contributions to our knowledge of spirochetes, particularly in the difficult problem of their cultivation, reported in 1919 that yellow fever is caused by a leptospira. The evidence for this view was gathered with Noguchi's usual thoroughness and it was widely accepted until 1928, when Stokes, Bauer, and Hudson showed that yellow fever is caused by a filtrable virus. Noguchi had apparently worked in 1919 with cases of Weil's disease mistakenly diagnosed as yellow fever. He later went to Africa to check his own findings. This time he worked with yellow fever and did not find leptospires. While there Noguchi contracted yellow fever and died.

#### LEPTOSPIRA CANICOLA FROM A CULTURE

(On dark field) (160) *Leptospira canicola*, the leptospira of dogs, is morphologically identical with the other leptospires. It can be distinguished serologically. It is less pathogenic for guinea pigs, but may infect both dogs and man. The prevalence of canicola infection in dogs is suggested by the finding of Meyer and his co-workers that agglutinins to *Leptospira canicola* occurred in a high percentage of normal dogs in urban areas of California. The canicola infection in man, contracted from dogs, is milder than Weil's disease, and may be so mild as to escape detection. Jaundice is usually absent; meningeal symptoms may be present. Leptospires may be found in the spinal fluid or blood, later in the urine. By the seventh to the tenth day of illness or later, a diagnosis can be made by the agglutination of culture *Leptospira canicola* with the patient's serum.

#### SPIROCHETE-LIKE FILAMENTS: ARTIFACTS WHICH MAY BE MISTAKEN FOR LEPTOSPIRAS

(On dark field) (120) These filaments, seen here as delicate waving threads, sometimes attached to cells and sometimes free, are of no importance in themselves. They are not microorganisms. They are shown because they may resemble microorganisms, especially leptospires, and thus give rise to errors in diagnosis. Although they are often homogeneous, they may show irregular swellings, clubbed ends, or beading. Occasionally they have a regular beaded structure, as shown here, which may give a false impression of spirals. Filaments occur frequently in dark-field preparations made from blood or tissue emulsions, particularly but not only when a hot loop has been used in preparing the slide. They may be extremely delicate and pale, or coarser and brighter, and vary from short rods to long waving threads. They have no independent motility, do not rotate or move progressively; but they may wave and bend actively in Brownian movement. The two features that best distinguish them from leptospires are the absence of hooked ends and the lack of rotary movement. (Elapsed time 48 minutes, 25 seconds)

#### SPIRILLUM MINUS FROM THE BLOOD OF AN INFECTED MOUSE

(On dark field) (140) This organism, seen lying still among red blood cells, is a spirillum, not a spirochete. The difference, as we have seen, is that a

spirillum is a curved or spiral organism motile by means of flagella, while a spirochete moves, as far as we know, by means of the undulations of the body of the organism itself. Flagella have never been demonstrated on true spirochetes. They appear on these organisms, and you will see a tuft at each end in later sequences if you watch for them.

*Spirillum minus* appears under dark field as a short thick rodlike form, just barely double contoured, with rather closely set stiff spirals which are not always clearly visible.

(Wait 5 seconds) (110) This organism is generally regarded as the cause of rat-bite fever or Sodoku. Since its cultivation has not been successful, it has not been possible to fulfill Koeh's postulates for *Spirillum minus* as the etiologic agent of rat-bite fever; however, inoculation experiments with unpurified animal blood or exudate have been successful in animals and in man. The relationship of this organism to rat-bite fever has been challenged in favor of a distinct bacterium called *Streptobacillus moniliformis*, which may also occur in the human lesions and in infected rats and mice, and which can be cultivated independently.

(Wait for flagellated form) Flagella can be seen on this organism and in several of these sequences. (Wait for another flagellated form) Note the tuft of flagella at each end (and another), and here again.

#### SPIRILLUM MINUS IN PERITONEAL WASHINGS FROM AN INFECTED MOUSE

(On dark field) (120) When *Spirillum minus* moves, it darts rapidly across the field. You will see these darting movements in a little while.

(Wait 5 seconds) (130) Whether or not this organism is the cause of rat-bite fever, diagnosis can be made if it can be demonstrated under the dark field in exudate expressed from the bite wound or from the skin papules which accompany the paroxysms of fever. The bite wound usually heals during the incubation period, and later ulcerates and looks like an extragenital chancre. The demonstration of spirilla in the blood or peritoneal fluid of mice or rats after inoculation with the patient's blood is less reliable for diagnosis, since these animals may harbor the organism normally without showing symptoms. The subcutaneous inoculation of guinea pigs is probably a better procedure. Numerous instances of clinically typical rat-bite fever fail to yield spirilla. *Streptobacillus moniliformis* has been isolated from some of these. It seems likely that the streptobacillus is responsible for some cases, at least, of rat-bite fever. Recent findings suggest that there may be two types of the disease, clinically similar, although perhaps not identical, but caused by distinct organisms. (Elapsed time 52 minutes, 25 seconds)

(On apparatus) (120) The apparatus includes a rigid stand supporting the camera independently of the microscope, the two being joined without contact by a light-tight coupling. A prism side ocular permits visual examination of the field being photographed. In order to allow one-man operation, a solenoid mounted beside the camera release is actuated by a foot switch, as shown here.

Pure strains of spirochetes were provided through the courtesy of:

Venereal Disease Research Laboratory, Marine Hospital, Stapleton, S. I.: *T. pallidum* (Nichols strain).

Dr. Thomas B. Turner, Department of Bacteriology, School of Hygiene and Public Health, Johns Hopkins University: *T. recurrentis* (novyi strain); *T. cuniculi* (strain A); *L. icterohemorrhagiae* (human strain S. E.); and *Spirillum minus*.

Dr. Laura Florence, Department of Bacteriology, New York College of Medicine: *L. biflexa*.

Dr. Elberton J. Tiffany, Department of Bacteriology, Long Island College of Medicine: *L. canicola* (strain D95). (Total elapsed time 54 minutes)

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# A SIMPLIFIED APPARATUS FOR DARK-FIELD CINEMICROGRAPHY\*

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THE apparatus described is that used for the preparation of the 16 mm. film "A Lecture on the Spirochetes."<sup>1</sup> Except for the microscope and camera, it comprises few and inexpensive items. It is designed to be operated by one person, and is arranged so that with a minimum of inconvenience records on motion-picture film may be made of specimens observed during routine visual microscopy. The apparatus is adaptable to bright-field, histologic or other microscopy, as well as to dark-field microscopy with the oil-immersion objective, although it was designed primarily for the purpose last mentioned.

The optical system used comprises a Leitz AT microscope with the fluorite oil-immersion objective, 1.95 mm., N.A. 1.32, fitted with an iris diaphragm, and the Leitz bicentric dark-field condenser, N.A. 1.20. The focusing prism side ocular (Fig. 1 A) is similar to that supplied as the optical portion of a Leitz Makam camera, the camera box and ground glass having been removed, and the lower element of a 10× ocular, convex surface upward, having been fastened immediately above the prism in the optical axis to shorten the focal length of the system. A celluloid shield, blackened and lined at the outer edge with black felt fitted to the eye, is attached to the end of the lateral member of the side ocular so that when the operator's eye is pressed against it extraneous light is excluded. No camera lens is used. The camera, which is supported independently of the microscope, as will be noted below, is joined to the optical system without contact by means of a light-tight brass baffle coupling (Fig. 2H), threaded to fit into the camera in place of a lens. The inner surface of the brass coupling must be dead black. A cylinder projecting upward from the top of the side ocular unit (Fig. 2J) fits into the coupling without contact, so as to make the light-tight baffle connection between the microscope and camera.

The whole assembly is fastened to a large heavy draftsman's board, which in turn stands on four No. 7 live rubber stoppers to absorb vibration (Fig. 3). The microscope is mounted on a steel plate which is bolted to the board and cushioned with live rubber washers  $\frac{1}{4}$  inch thick. The holes in the plate through which the bolts pass are large enough so that the bolts do not touch the steel plate itself, but are held by rubber washers above and below the plate. The plate is fitted with three steel pegs which mate accurately in shallow holes in the base of the microscope.

The stand upon which the camera is mounted consists of a heavy cast iron base, a tubular steel post (Fig. 1B), and an adjustable bracket with ball crank (C), which are available as parts of a standard drill press. Firmly attached to the bracket is a carriage (D) bearing a tripod screw and its bushing, and with side guides made to support the camera with a snug fit. At the outer end of

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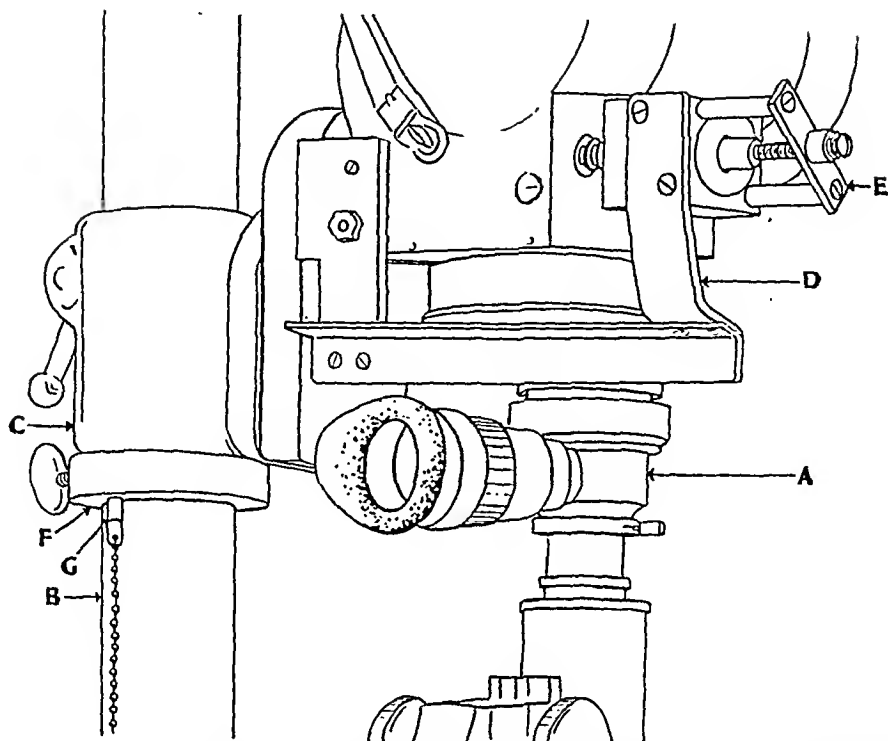


Fig. 1.—A, Prism side ocular; B, post supporting camera; C, adjustable bracket; D, camera carriage; E, electric solenoid camera release; F, collar; G, pin; F and G serve respectively as vertical and horizontal stops for the bracket and camera carriage.

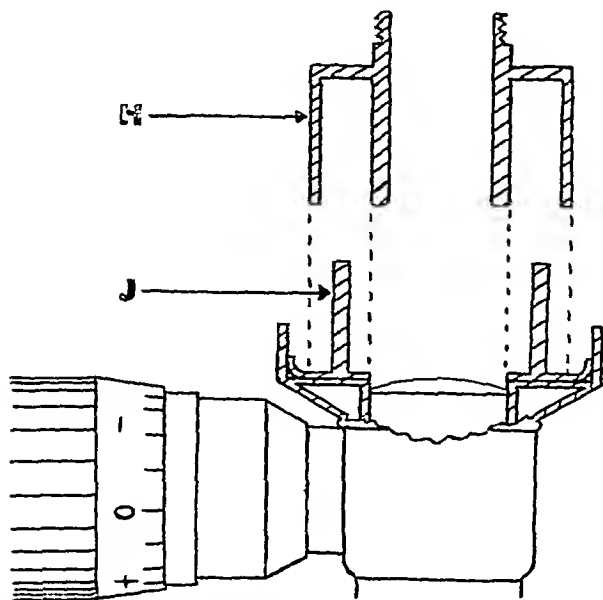


Fig. 2.—Detail of light-tight baffle joint between the camera and the prism side ocular, with the brass coupling (H) shown raised out of position above the cylinder (J).



the carriage is mounted an electric solenoid (*E*), the magnetic plunger of which is normally retained in a position on the outer side of the coil by means of an adjustable spring. When current is applied by means of a foot switch the plunger presses the release button of the camera. The end of the plunger carries a rubber bumper.

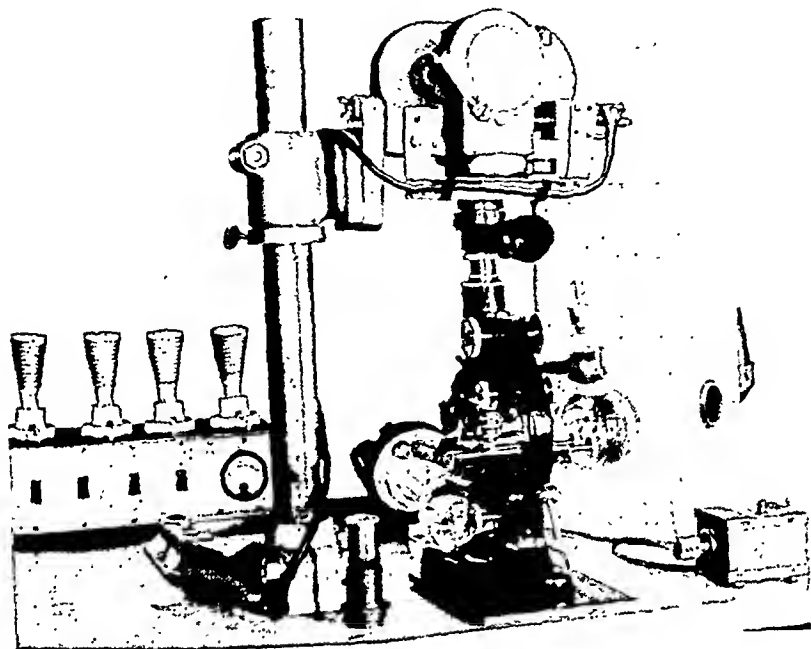


Fig. 3.—Photograph of the entire assembly.

A collar (Fig. 1*F*) is fastened to the post with a setscrew to serve as a vertical stop for the camera assembly and its bracket on the post. Horizontal locating is determined by means of a spring pin (*G*) in the collar, which fits accurately into a hole in the adjustable bracket. The spring pin is released by drawing down the chain attached to it. By this means the camera assembly can be lowered quickly into its proper position over the microscope and prism side ocular.

Two light sources are used, one for visual microscopy by bright or dark field and for bright-field photomicrography, the other for dark-field photomicrography. The former is a 6 volt automobile headlight lamp in a parabolic mirror reflector, operated on 110 volts A. C. through a variable voltage transformer (Bausch & Lomb). The stronger light source is a clockwork are adapted to take National Projector carbons Nos. 698 or 722, ( $\frac{7}{16}$ " ) and 168 ( $\frac{5}{16}$ " ). The arc is operated on 110 volts D.C. through four 660 watt cone heater elements connected in series parallel with a switch in each parallel branch so that the arc current, which is read on an ammeter, may be conveniently adjusted in four steps. The resistances, switches, and ammeter are mounted on a transite and metal stand.

Both lamps are fastened in position on the draftsman's board so that their rays, condensed and cooled in each instance through a 250 c.c. flask of water,

are focused on the mirror of the microscope when the latter is in position on its steel plate. This provides a convenient arrangement for visual microscopy with the 6 volt lamp. When a specimen is seen which it is desired to photograph, the split prism with its ocular is substituted for the visual ocular of the microscope. The camera, with coupling attached, is swung and lowered into position with the aid of the collar and pin on the post and clamped tight, and the are is turned on. With these operations, which require less than one minute, the apparatus is converted from a visual to a cinemicrographic assembly.

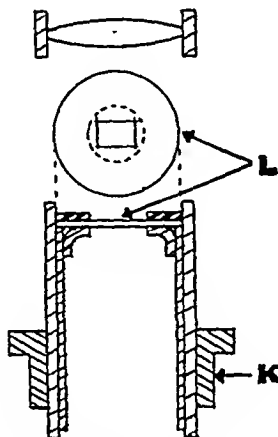


Fig. 4.—Focusing calibrator. *K*, Turned collar fitting into cylinder *J* in Fig. 2; *L*, cover slip ruled to 16 mm. frame size and placed in the focal plane.

The calibration of focus for the visual arm of the split prism ocular and the film was accomplished by means of the device shown in Fig. 4. A fiber cylinder 2 inches high was fitted with a turned wood collar (*K*) so as to set snugly into the baffle cylinder (Fig. 2*J*) of the split prism ocular. A clear circular cover slip (Fig. 4*L*) was then ruled with hydrofluoric acid to approximate a 16 mm. frame, and fastened in position in the cylinder at the level which would be occupied by the film when the camera was in place, as determined by measurement. A focusing magnifier could then be set into the cylinder from above so as to focus on the ruled cover slip. With this device the focus at the film plane could be matched with that in the visual arm of the split prism by varying the length of the latter. This calibration was checked by exposing and developing short lengths of film.

The film "A Lecture on the Spirochetes" was made chiefly on Agfa Superpan Supreme negative, which provided sufficient speed with maximum amperage of the arc (15 to 20 amperes). Photographs of specimens containing large numbers of cells, e.g., red blood cells, were in fact overexposed, although printable; and several sequences were made successfully with a slower film, DuPont 314 Pan. The film was developed, using Stineman tanks, in Agfa 17M at 18 C. for sixteen minutes with continuous agitation ( $\gamma = 0.9$ ). Under these conditions no objectionable grain appeared. Resolution on the negative seems remarkably good at the low magnification used (160 diameters), and, although slightly impaired in printing, it is considered satisfactory in the finished film.

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# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**DIPHTHERIA**, Studies on the Antibacterial Property in, Huang, C. H. *Am. J. Hyg.* 35: 317, 1942.

Evidence pointing to the presence of antibacterial property in the diphtheria antitoxic serum is presented, based on the finding of the rapid disappearance of type D<sub>2</sub> organisms in the throats of patients during the first two weeks after treatment with antitoxic serum as compared with the slow disappearance of the organisms in the throats of patients infected by the other S types. Type D<sub>2</sub> organism is serologically the same as Park S strain which is universally used for the preparation of diphtheria antitoxin.

Specific agglutinins were found in the blood of diphtheria patients during the convalescent stage. In the acute stage such agglutinins were not found. A negative agglutination test with the blood of a diphtheria patient at the early stage of the disease and the appearance of specific agglutinins in the convalescent stage may, therefore, serve as a diagnostic method.

**TARRY STOOL**: Observations on the Oral Administration of Citrated Blood in Man. II. The Effect on the Stools. Schiff, L., Stevens, R. J., Shapiro, N., and Goodman, S. *Am. J. M. Sc.* 203: 409, 1942.

A tarry stool may result from the ingestion of 100 c.c. or more of citrated human blood. Following the intragastric administration of 1,000 to 2,000 c.c. of citrated human blood, gross blood may appear in the stools, and tarry stools may continue for five days and may number as many as 8. Positive chemical tests for occult blood in the stools may persist for as long as ten days after ingestion of 250 c.c. of citrated human blood or for twelve days after intragastric administration of 2,000 c.c.

The passage of a tarry stool does not necessarily indicate the occurrence of a severe hemorrhage into the digestive tract. The persistence of tarry stools or occult blood in the stools does not necessarily indicate the continuation of such hemorrhage.

**CULTURES**, A Note on the Preservation of Certain Bacteria Under Paraffin Oil. Simons, R. T. *M. J. Australia* 1: 283, 1942.

Overnight slope cultures were completely covered with sterile liquid paraffin (heavy mineral oil; Parke, Davis Company) and stored in a dark cupboard at room temperature. The slopes should be moist, and they should be inoculated to the bottom of the slope so that some bacteria are suspended in the water of condensation which remains at the bottom of the slope under the paraffin. The paraffin was sterilized by dry heat at 160° C. in amounts of 15 milliliters heated for one hour, or in amounts of two liters heated for two hours. Autoclaving or the presence of moisture in the container caused turbidity. Paraffin from discarded cultures may be strained through gauze, sterilized and used again.

The top of the culture slope must be covered by the paraffin (preferably half an inch above) in order to prevent drying. A long, stiff, platinum loop was used to remove the growth from the bottom of the slope, where the organisms remained viable longer than at the top. After one year it was occasionally necessary to replace the paraffin with broth in order to obtain growth, but it was generally sufficient merely to transfer a loopful of organisms to broth.

**PLASMA COAGULATION TIME, The Normal, Cheney, G. Am. J. M. Sc. 203: 325, 1942.**

The plasma coagulation time is a satisfactory test for vitamin K deficiency, as previously noted.

The test has been carried out by a standardized technique in 340 normal persons and in 107 patients with nonhemorrhagic conditions.

The importance of a constant speed and time in centrifuging the blood has been emphasized.

The effect of changes in temperature upon the results of the test has been pointed out. Room temperature is satisfactory under ordinary conditions.

Standards have been set up for laboratory temperatures which may be encountered and for 37° C.

The constancy of the results obtained warrants the use of the plasma coagulation time as a simple laboratory test, provided the physical conditions of the procedure are adhered to closely.

The test of plasma coagulation time is routinely carried out as follows:

1. A clean venipuncture must be performed, obtaining the required amount of blood in a dry syringe.

2. The blood must be rapidly transferred to a test tube containing a weighed amount of dry potassium oxalate. A number of these tubes may be prepared at one time by adding 2 per cent potassium oxalate and evaporating the solution so that the tube will contain oxalate in the ratio of 10 mg. to 5 c.c. of blood. Centrifuge tubes are usually prepared with 5 mg. of oxalate for 2.5 c.c. of blood (0.25 c.c. of the 2 per cent solution per tube). The oxalate must be thoroughly mixed with the blood by inverting the corked tube ten or fifteen times.

3. The oxalated blood is centrifuged in a constant speed centrifuge at 3,500 r.p.m. for five minutes and the plasma is pipetted off.

4. Two-tenths cubic centimeter of plasma is pipetted into each of two small chemically clean test tubes, and 0.2 c.c. of 0.4 per cent calcium chloride is added to the first tube and 0.1 c.c. to the second. The tubes should be gently shaken five to ten times to secure thorough mixing. They need not be inverted.

A liter of the 0.4 per cent calcium solution may be made up at a time, using chemically pure calcium chloride.

5. The coagulation time of the plasma should be read in minutes. The end point is taken when the solution no longer flows when the test tube is held horizontal. The shortest time of coagulation for the two tubes is the plasma coagulation time.

6. The room temperature at which the test is carried out should be recorded. A variation between 23° and 26° causes no gross variation in the test.

7. The test should be completed within the first hour after the venipuncture.

**CLOT RETRACTION TIME, A Method for Measuring, Hirschboeck, J. S., and Coffey, W. L. Arch. Path. 33: 380, 1942.**

The equipment needed is a clean, dry syringe with needle and two dry test tubes, 1 cm. in diameter, one of which serves as a control. The tubes should be of soft glass and must be free of scratch marks. In addition they must be chemically clean. With the syringe and needle 4 c.c. of venous blood are removed, and 2 c.c. are placed in each of the test tubes. The tubes are closed with rubber stoppers, and the blood is shaken for a few seconds in order to create an equal amount of foam in each tube. The coagulation time is then determined by measuring the interval of time between the complete withdrawal of the blood from the vein into the syringe and the point at which the blood fails to flow when the tubes are completely inverted. The tubes are held in the same hand and are tilted every half minute in order to test for the end point. The end point of coagulation then becomes zero for the measurement of the retraction time. The tubes are placed in a rack kept at room temperature and are observed for the beginning of retraction.

The normal clot retraction time with this method is usually between twenty-five and thirty minutes. Some normal bloods may have a retraction time as short as twenty minutes; others, as long as forty-five minutes. The clot retraction time becomes shortened postoperatively, and if it is less than ten minutes, the patient may be considered a possible candidate for pulmonary embolism.

**ANTITHROMBIN, Quantitative Studies on, Wilson, S. J.** Arch. Int. Med. 69: 647, 1942.

A quantitative test has been devised for the determination of the antithrombin of serum and plasma. One unit of antithrombin is defined as that amount which will inactivate 1 unit of thrombin in four minutes at 28° C.

Normal human plasma or serum contains an average of 90 units of antithrombin per cubic centimeter, which is as much as a thousand times the amount revealed by many previous methods.

There is little or no difference in the antithrombic activity of serum and that of plasma.

During the actual process of blood coagulation only a small portion of the thrombin is adsorbed onto fibrin, the remainder being inactivated or neutralized by the normal antithrombic activity of the serum.

The hemorrhagic tendency in hypoprothrombinemia is not, therefore, fully explained on the basis of decreased prothrombin and the variations in the conversion rate of prothrombin to thrombin. The hemorrhagic diathesis usually occurs when the prothrombin unitage approximates or is lower than the antithrombin unitage.

The method follows:

The prothrombin-free fibrinogen was prepared by the method of Warner, Brinkhous, and Smith, all procedures being carried out at 5° C. and the materials stored at -35° C. The thrombin was prepared by the method of Seegers, Brinkhous, Smith, and Warner and standardized by either the pipette or the dropper method. The standardization tests should be repeated just before any critical observations are made.

The following method was devised to determine accurately fractions of 1 unit of thrombin: Fifteen drops of physiologic solution of sodium chloride were added to each of 9 test tubes. A series of 9 dilutions, containing 0.2 to 1.0 unit of thrombin in each drop, was then prepared, and portions of a single drop were added in sequence to the tubes of saline solution. To each test tube was then added 5 drops of fibrinogen (a total of 21 drops), and the appearance of the fibrin strands was timed with a stop watch. The following clotting times were observed:

| Units of Thrombin | Clotting Time, Seconds |
|-------------------|------------------------|
| 1.0               | 20                     |
| 0.9               | 21.5                   |
| 0.8               | 23.5                   |
| 0.7               | 25.5                   |
| 0.6               | 27.5                   |
| 0.5               | 31                     |
| 0.4               | 35.5                   |
| 0.3               | 40.5                   |
| 0.2               | 46.5                   |

A curve to be used in the studies on quantitative antithrombic activity was then plotted. One unit of thrombin was used in the studies. If the figures for the thrombin are reversed, the curve will indicate the amount of thrombin inactivated or neutralized.

**HEPATIC DEFICIENCY, The Value of the Hippuric Acid Test and Takata-Ara Reaction in the Investigation of, Henderson, M., and Splatt, B.** M. J. Australia 1: 185, 1942.

A series of cases is reported of 313 hippuric acid excretion tests performed on 240 adult subjects.

The serum of 143 patients and of 50 normal subjects has been tested by the Takata-Ara reaction.

Quick's hippuric acid excretion test is both a reliable and a sensitive index of liver function, and is a valuable aid in estimating clinical progress, response to treatment, and prognosis.

It is simple to perform and is devoid of danger to the patient, but is not applicable to patients with poor renal function.

The Takata-Ara test is practically valueless as a test of liver function and is not a specific test for liver disease. It is, however, a useful confirmatory test for parenchymatous liver disease.

**PREGNANCY, Colostrum Cutaneous Test for, Goldmann, L. M., Kessler, H. B., and Wilder, M. E. J. A. M. A. 119: 130, 1942.**

In 500 tests of cutaneous sensitivity to colostrum there were tested approximately 70 per cent correct reactions in all groups, both male and female.

There was apparently no greater or less efficiency of the test among endocrine cases.

In our hands the colostrum cutaneous test does not appear to offer a valuable diagnostic procedure for pregnancy.

**AMINO-ACID, Plasma Levels in Health and in Measles, Scarlet Fever and Pneumonia, Farr, L. E., McCarthy, W. C., and Francis, T., Jr. Am. J. M. Sc. 203: 668, 1942.**

Thirty-one determinations of the plasma amino acid concentration of 30 normal, apparently healthy persons averaged 4.50 mg. per 100 c.c. by the ninhydrin- $\text{CO}_2$  method. The standard deviation was 0.46, and the observed range was 3.75 to 5.56 mg. per 100 c.c.

In patients with pneumococcus pneumonia the plasma amino acid nitrogen concentration was found to be low at the time of onset. With recovery from pneumonia the plasma amino acid concentration returned to the normal range. Patients admitted to the hospital after the fourth day of disease failed to show significant plasma hypo-amino-acidemia.

In patients with scarlet fever and measles the plasma amino acid nitrogen concentration was usually within the normal range. However, because of the difficulty of ascertaining the time of onset of these diseases, the data do not exclude the possibility of a plasma hypo-amino-acidemia during the pre-eruptive phase of these diseases.

**PLASMA PROTHROMBIN Concentration, Clinico-Pathologic Correlation Between Hepatic Damage and, Sweet, N. J., Lucia, S. P., and Aggeler, P. M. Am. J. M. Sc. 203: 665, 1942.**

The prothrombin concentration was determined in 42 patients in whom the liver was examined at operation or at autopsy. Vitamin K was administered in adequate dosage to all those patients who had suffered from extrahepatic biliary obstruction. In general, the prothrombin concentration was normal when there was little or no destruction of the parenchymal tissue, and it was diminished when there was moderate or marked destruction of tissue. Exceptions to this rule are noted.

**TUBERCLE BACILLI, Rapid Method for Concentration of, Oliver, J., and Reusser, T. R. Am. Rev. Tuberc. 45: 450, 1942.**

Five to 10 c.c. of sputum are mixed in a flask or widemouthed bottle, with approximately an equal amount of commercial chlorox, and shaken two or three times over a two-minute period and kept at room temperature for ten minutes. Then the mixture is centrifuged at 3,000 r.p.m. for ten minutes in a 15 c.c. conical tube. A creamy white sediment forms in the bottom of the test tube; the supernatant fluid is poured off and the tube is drained for two minutes. No neutralization is necessary. With an applicator a small drop of sediment is transferred to a slide. The slide dries at once and needs no fixing. Staining is done by the usual methods.

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## CLINICAL AND EXPERIMENTAL

### PREVENTION OF PORPHYRIN INCRUSTATIONS ON PANTOTHENIC ACID-DEFICIENT RATS BY HARDERIAN GLAND ABLATION\*

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ONE of the most conspicuous symptoms observed in rats fed a diet deficient in pantothenic acid was the accumulation on the nose of an incrustated material that superficially resembled dried blood. This material appeared to emanate from the nostrils and was, therefore, observed most abundantly on the nose, whiskers, and fore paws, but it was sometimes spread to the fur of other parts of the body. The fur on the back of the neck region frequently appeared red or rusty. Because of the strong resemblance to dried blood and the apparent origin from the nose, this symptom had been described variously as "nosebleed,"<sup>7,8</sup> "blood-eked whiskers,"<sup>11</sup> "rusty spots on fur,"<sup>9,12</sup> and "inflamed noses."<sup>12</sup>

Several months before the initiation of the work to be reported in this paper, it was observed in collaboration with others<sup>10</sup> that the red material on the rats exhibited a brilliant red fluorescence when illuminated with near ultraviolet light. Since blood is not fluorescent, an attempt was made to characterize this incrustated red fluorescent exudate chemically, and to discover its origin.

The red fluorescence made it appear probable that the incrustated material was a porphyrin (see Plate I, page 1608). Chemical and spectroscopic examination proved this to be the case. Other chemical tests indicated that very little, if any, of the fluorescent material was blood.<sup>10</sup> A survey of the literature re-

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This work was aided by a grant from the Anna Fuller Fund for Cancer Research. The illustration for this article will be found in the section on Medical Illustration of this number of the JOURNAL, Plate I, page 1608.

†Rockefeller Foundation Fellow, 1940-1941.

Received for publication, March 1, 1941.

vealed that the reddish incrustated exudate which appears on rats maintained on a filtrate factor deficient diet had been identified spectroscopically and described as protoporphyrin. The tests for blood were also negative.<sup>2</sup> Our own independent observations and more exhaustive investigation confirm this observation in some respects.<sup>10</sup>

At this stage of the investigation a short abstract appeared describing the harderian gland as a porphyrin-secreting gland.<sup>6</sup> The fact that the harderian gland was fluorescent and contained protoporphyrin had been known for some time.<sup>4, 16</sup> It thus appeared likely that the material was secreted by the harderian gland and passed to the nose via the nasolacrimal duct. In order to test this hypothesis, the harderian gland was completely removed from a number of rats to see if this operation would prevent the accumulation of the porphyrin-containing incrustations (*see* Plate I, page 1608). These operated animals and an equal number of controls were maintained on a diet deficient in pantothenic acid. This experiment demonstrated convincingly that harderian gland ablation prevents the porphyrin incrustations in albino rats maintained on a diet deficient in pantothenic acid. It is the purpose of this paper to present the details of this experiment.

#### METHODS AND MATERIALS

The 24 albino rats used in the experiment were weaned at the age of 22 to 24 days. They were from three litters<sup>3</sup> and, in so far as possible, the animals from each litter were uniformly distributed in four groups. There were 12 females and 12 males so that 3 of each sex were placed in each group of 6 rats (*see* Table I). The males and females were separated and placed three in a cage. The cages were made of pyrex glass and prevented contact of the animals or their excreta with metal. The feces, urine, and spilled food fell between the glass bars to pyrex glass plates and could not be retrieved by the rats. The feces were examined periodically to see whether they contained a red fluorescent material. The weights of the rats and other observations were recorded bi-weekly.

All rats were fed a basic diet of cerelese, 68 per cent; vitamin-free casein, 18 per cent; crisco, 8 per cent; salt mixture, 4 per cent; cod-liver oil, 2 per cent. This basic diet was supplemented for all rats by the addition of choline chloride (1 Gm. per kilogram) and the following vitamins: thiamine† (4 mg. per kilogram), riboflavin† (5 mg. per kilogram), pyridoxine† (4 mg. per kilogram), and nicotinic acid† (50 mg. per kilogram).<sup>18</sup> Twenty milligrams of pantothenic acid per kilogram were added to this for 12 animals maintained on a nondeficient diet. These mixtures were pounded compactly into the food cups to prevent excessive spilling. During the experiment special care was exercised to keep the water bottles filled at all times. This is an important requirement when investigating the porphyrin-containing incrustations.<sup>5</sup>

The harderian gland was removed from 11 rats (*see* Table I); five of these in one group were fed a diet deficient in pantothenic acid and 6 rats in another

\*Obtained from the rat colony of the anatomy department through the courtesy of Dr. Charles W. Hooker.

†These vitamins were very kindly supplied by Dr. Robertson of Merck & Co.



group were fed the diet containing pantothenic acid. Rat 7 was not operated upon because at the time of operation it was thought that the animal would not survive the anesthesia. This unoperated animal did survive and was later regarded as a control for the two operated animals kept in the same cage.

The Harderian gland in rodents is very large, occupying in the case of the rat about one-half of the total volume of the orbital cavity. This gland is a lacrimal gland. It is associated with the nictitating membrane and, therefore, is located on the medial side of the orbit. The location appears to be the chief characteristic used to distinguish it from the true lacrimal gland which is either extra-orbital or on the lateral side of the orbit. The Harderian gland is horse-shoe-shaped and almost completely surrounds the optic nerve and bulbus oculi.

TABLE I

INFLUENCE OF HARDERIAN GLAND ABLATION ON THE ACCUMULATION OF THE ENCRUSTED RED FLUORESCENT EXUDATE IN RATS

|                   | ANIMAL |     | PORPHYRIN<br>ENCRUSTATION | CONDITION OF FUR            |
|-------------------|--------|-----|---------------------------|-----------------------------|
|                   | NO.    | SEX |                           |                             |
| Group 1A          | 1      | F   | Marked                    | Stained and matted          |
| Not operated      | 2      | F   | Marked                    | Stained and matted          |
| Pantothenic acid  | 3      | F   | Marked                    | Stained and matted          |
| Deficient diet    | 4      | M   | Marked                    | Stained and matted          |
|                   | 5      | M   | Marked                    | Stained and matted          |
|                   | 6      | M   | Marked                    | Stained and matted          |
| Group 1B          | 7*     | F   | Marked                    | Stained and matted          |
| Operated          | 8      | F   | None                      | White and matted            |
| Pantothenic acid  | 9      | F   | None                      | White and matted            |
| Deficient diet    | 10     | M   | None                      | White and matted            |
|                   | 11     | M   | None                      | White and matted            |
|                   | 12     | M   | None                      | White and matted            |
| Group 2A          | 13     | F   | Slight                    | Stained and fluffy          |
| Not operated      | 14     | F   | Slight                    | Stained and fluffy          |
| Pantothenic acid  | 15     | F   | Slight                    | Stained and fluffy          |
| Nondeficient diet | 16     | M   | None                      | Stained at times and fluffy |
|                   | 17     | M   | None                      | Stained at times and fluffy |
|                   | 18     | M   | None                      | Stained at times and fluffy |
| Group 2B          | 19     | F   | None                      | Very white and fluffy       |
| Operated          | 20     | F   | None                      | Very white and fluffy       |
| Pantothenic acid  | 21     | F   | None                      | Very white and fluffy       |
| Nondeficient diet | 22     | M   | None                      | Very white and fluffy       |
|                   | 23     | M   | None                      | Very white and fluffy       |
|                   | 24     | M   | None                      | Very white and fluffy       |

\*Nonoperated.

The operations were performed with sodium amytal anesthesia (0.1 mg. per gram of rat weight). If necessary, this was supplemented with ether, but this practice has little to recommend it. The ether caused some animals to develop mucous plugs which gave rise to respiratory difficulties. In the first 3 animals two incisions were made between the eyes near the medial angles. The lateral edge was pulled laterally to expose the medial margin of the orbit. After the fascia attached to the medial margin of the orbit was cut, the gland was exposed. A suction pipette was applied to pull the gland out of the orbit and at the same time the blood vessels were cauterized. The blood vessels and other connections were then cut with scissors. During the operation the field was illuminated alternately with visible and near-ultraviolet light. This not only facilitated

locating the gland, but also insured the complete removal, because even a tiny bit of gland was so highly fluorescent that it was very conspicuous in near-ultraviolet light. A small portion of a harderian gland was found in the orbit on only one operated animal when the rats were killed and examined at the end of the experiment.

During the first few operations it was found that in order to remove completely the gland, the nictitating membrane and conjunctiva were frequently cut and damaged. In view of this, it appeared superfluous to make an incision in the skin. The last sixteen glands were removed by cutting the conjunctiva near the medial angle of the eye with an iridectome. The nictitating membrane and attached harderian gland were removed together. The other connections of the gland were cut with scissors, but no attempt was made to use a cautery. With this method the animals lost a drop or two of blood, but the eyes soon returned to normal. Since no incision was made in the skin, and no suturing was required, this was a much more rapid method. Examinations at the end of the experiment revealed only one eye damaged, and this was in one of the first animals operated upon. This injury was thought to have been caused by the cautery. Only one animal (No. 24) was blinded by the operation; the eyes of the other operated animals were normal. It was thought that all the animals would be blind from conjunctivitis caused by drying of the conjunctiva. The eyes of the operated animals were apparently kept sufficiently moist by the secretions from the extra-orbital lacrimal gland. This extra-orbital gland, located just in front of the ear, was found to exhibit a slight red fluorescence in only 6 of 50 rats examined specifically for this. No mention of the variable red fluorescence which was observed in this extra-orbital lacrimal gland was found in the literature. Even though there may have been some porphyrin in the secretion of some of these glands, it was never observed on the nose or fur of any of the animals with the harderian gland removed.

#### RESULTS

The experimental conditions for the different groups of animals and the observations regarding the porphyrin incrustations and condition of the fur were tabulated (see Table I). No fluorescent porphyrin-containing incrustations were observed at any time on animals in which the harderian gland had been removed. This was true regardless of the diet (group 1B, 2B). All non-operated animals maintained on a diet deficient in pantothenic acid showed fluctuating, but marked, porphyrin incrustations (group 1A). These incrustations were red fluorescent and were most abundant on the nose, whiskers, and near the medial angle of the eye (see Plate I, page 1608). These symptoms required about four to five weeks to appear, and after this time varied from time to time, but in general became increasingly severe. Some of the nonoperated animals, maintained on a diet not deficient in pantothenic acid, exhibited slight and variable porphyrin incrustations. The slight porphyrin incrustations in this group were observed most frequently on the females. Operated animals maintained on a similar diet not deficient in pantothenic acid appeared unusually white and clean (group 2B) throughout the experiment. These very clear-cut

results indicate that the harderian gland is the source of the porphyrin-containing red fluorescent incrustations.

In addition to these observations several others were also recorded. An intense red fluorescence was observed in the feces of all nonoperated animals. In the feces of operated animals no red fluorescence was observed. The weight curves for the animals on the deficient diet were considerably below those on the nondeficient diet. Since these weights were found to agree with those of other investigators,<sup>18</sup> they have not been reproduced. The fur on all animals fed a diet deficient in pantothenic acid appeared matted. Depilation of body fur and bald head also occurred in some animals. The removal of the harderian gland did not influence these symptoms.

#### DISCUSSION

Previous investigations<sup>4, 6, 10</sup> had established the fact that the harderian gland contained and secreted porphyrin. The physiologic significance of the porphyrin in this fluid, which bathes the conjunctiva, has not been discovered. The anatomical pathway of this harderian gland secretion was followed by observing the red fluorescence. Under normal conditions the secretion was found to collect in the sacculus lacrimalis and pass to the nasal cavity by way of the nasolacrimal duct. A fluorescent nasolacrimal duct was found in several cases. From the nose it may pass either to the alimentary tract directly via the nasopharynx or to the nose and whiskers through the external nares. In a normal rat on an adequate diet some of this red pigment was seen occasionally in visible light on the fur. When these normal rats were irradiated with near-ultraviolet light, a small amount of this red fluorescent material was frequently seen near the external nares. It was usually not very conspicuous, however, as healthy rats keep it cleaned off by washing and licking.

In some rats porphyrin incrustations were induced by water deprivation. When these rats were given water, they drank it and soon cleaned the incrustated material off by licking and washing. The rats were killed two to four hours later. A red fluorescent band was observed in the intestine. It was assumed that this band was caused by the unusually heavy dose of concentrated porphyrin cleaned off the nose and fur by washing. This may account for the presence of large amounts of porphyrin which had been observed in the excreta of rats.<sup>13</sup> The red fluorescence of the feces was not observed in the case of the operated animals. This is no proof, but it suggests that the harderian gland may elaborate the porphyrin which it secretes.

In the case of nonoperated rats, maintained on diets deficient in pantothenic acid, this porphyrin-containing secretion accumulated and became incrustated on the nose, whiskers, and face. The infrequent and feeble attempts to remove this by washing and licking were unsuccessful and usually the material was merely smeared around to other areas, notably the fore paws and back of the neck. This smearing and unsuccessful cleaning, while partly caused by the lethargy of the rat, was also thought to be related to a change in the physical nature and the amount of the saliva. It appeared to function like a liquid glue that did not dissolve or remove the material, but acted as an adhesive base for the smeared porphyrin-containing excretion.

Much evidence was found to support this concept of the mechanism of the accumulation of porphyrin incrustations. Water deprivation and limitation induced porphyrin incrustations in rats.<sup>5</sup> This had been observed previously, but the red exudate was not only mistaken for blood, but the tests for blood were positive.<sup>15</sup> Pantothenic acid deficiency may influence water metabolism indirectly through its effect on the adrenals. A deficiency of this vitamin in the diet caused adrenal cortical damage and necrosis.<sup>1, 3, 11, 14</sup> Experimental adrenal cortical insufficiency resulted in the loss of water from the plasma and general dehydration as indicated by hemoconcentration in cats and decreased plasma volume in dogs.<sup>8</sup> The rats fed a deficient diet sneezed more frequently and drank less water (approximately one-half as much) than the animals on a nondeficient diet. It was thought that this sneezing might be an indication of a relatively dry nasal mucous membrane.

The porphyrin-containing exudate which was seen to come from the nose was doubtless a mixture of the harderian gland and nasal secretions. Any changes in physical properties of these secretions that would result from a general dehydration would also have to be regarded as an important factor in the control of the adhesive properties of this exudate. From these bits of evidence gathered from various sources, it may be seen that pantothenic acid deficiency caused adrenal necrosis<sup>1, 3, 11, 14</sup> which, according to the work of Lewis,<sup>8</sup> caused a loss of water from the plasma and general dehydration. Water deprivation and consequent dehydration caused a marked decrease in salivary flow in man and dogs,<sup>7</sup> and both pantothenic acid deficiency and water deprivation induced the porphyrin incrustation symptom in rats.<sup>5</sup> These facts indicate that pantothenic acid deficiency regulates water metabolism; it suggests the mechanism by which it does this, and influences the accumulation of porphyrin incrustations.

#### SUMMARY

Ablation of the harderian glands prevents the accumulation and incrustation of a red fluorescent porphyrin-containing exudate in rats fed a diet deficient in pantothenic acid. This indicates that the harderian gland is the immediate glandular source of the porphyrin in the incrustated material observed on the nose, whiskers, and fur of rats fed a diet deficient in pantothenic acid.

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## DUODENAL BULB ("ULCER-BEARING AREA") ACIDITY IN FASTING NORMAL PEOPLE\*

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IN THE present-day concept of the problem of chronic peptic ulcer the acid gastric juice occupies a position of acknowledged prominence. Since the gastric acid is important only because of its effects, direct and indirect, either on the ulcer proper or on some area susceptible to ulcer, one wonders why attention has been directed so exclusively to acidity in the stomach when most clinical ulcers occur in the duodenum.<sup>4</sup> Simultaneous determinations of gastric and duodenal acidity would serve better to evaluate the accepted importance of the acid gastric juice in duodenal ulcer. However, our knowledge of the changes in acidity which occur in the "ulcer-bearing" duodenal bulb is very limited.<sup>16, 26</sup> We particularly need more extensive information concerning the situation which normally exists in the duodenal bulb before we can hope to interpret the findings in ulcer patients.

In a previous study<sup>5</sup> we found, in agreement with Morton,<sup>26</sup> that the contents of the duodenal bulb in normal people following an Ewald meal were endowed with a considerable capacity to neutralize, buffer, and dilute the gastric chyme. Several investigators,<sup>13-15, 19, 25-27</sup> using men as subjects, had commented on the apparent lack of close relationship between gastric and duo-

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denal acidity and we were able to demonstrate a similar lack of sharp parallelism between the acidity in the stomach and that present at the same moment in the first part of the duodenum of normal persons after an Ewald meal.

The present study was undertaken to determine whether a neutralizing efficiency of equal magnitude and relationships of like nature obtained in the duodenal bulb during fasting in normal people studied under the same experimental conditions.

#### MATERIAL

The subjects utilized for the purposes of this study were drawn largely from the Out-Patient Gastro-Intestinal Clinic of the Jefferson Hospital. Although a number of them presented symptoms referable to the digestive tract, none gave evidence of fever or of any acute, chronic, infectious, metabolic or cachectic disease, and x-ray examination revealed no abnormality of the stomach and duodenum. Achlorhydries were not excluded, since a lack of free acid in the stomach is expected in a certain percentage of "normal" persons.<sup>2, 3, 20, 23, 24, 29, 30, 35</sup>

We could not hope to standardize all the variables which are known to affect gastric secretory behavior even in "normal" people<sup>8</sup> and, inasmuch as our primary concern was with the changes in acidity in the duodenal bulb which might be associated with changes in gastric acidity, we felt that variations in gastric acidity were actually desirable for our purpose.

Twenty-two subjects were studied, of which 14 were males and 8 were females. The age of the entire group ranged from 22 to 51 years, with an average age of 35.5 years. The males ranged from 22 to 51 years of age, with an average age of 32.5 years; the females from 29 to 49 years of age, with an average age of 40.9 years.

#### METHOD

Each subject was examined in the morning before breakfast, following a period of alimentary rest of at least twelve hours. A specially constructed double lumen tube was employed, and a method was utilized which permitted more or less fluoroscopic control as well as radiographic proof of the position of the tube.<sup>5</sup> Most of the subjects had swallowed tubes before for other purposes so that the element of fright was minimal. If much difficulty was encountered in swallowing the tube, if nausea or vomiting occurred, or if the subject complained of any other distress of any moment, the experiment was discontinued, and the results were disregarded. After ascertaining the location of the tube in the stomach with the aid of the fluoroscope, we were able, in some instances, to manipulate the tube into position in the duodenum. When we failed in this attempt, the subject was placed on his right side, the room was darkened, and the person was exhorted to relax and slowly swallow about 6 inches of the tube over a period of half an hour; not uncommonly the subject was asleep when we returned to the room at the end of that period.

Once the tube was determined by fluoroscopic examination to be in the desired position, the person was placed at rest on his right side, and the aspiration apparatus was assembled. Specimens were collected simultaneously from

the pars pylorica and the duodenal bulb at ten-minute intervals for one-half hour. At each of the four-interval aspirations all the material which could be obtained was collected.

The pH of each specimen was determined at once by means of a Leeds-Northrup pH indicator, which uses a glass electrode. After filtration the free and total acidity of each specimen was estimated using Töpfer's reagent and phenolphthalein as the respective color indicators.<sup>6</sup> In addition, on each duodenal specimen, we determined what was called the excess neutralizing ability.<sup>4, 6</sup> This consisted of the amount of N/10 hydrochloric acid necessary to lower the pH to the point at which Töpfer's reagent evidenced a positive reaction for free acid.<sup>6</sup>

Out of 29 experiments 25 were accepted as being satisfactory with respect to technique. These involved 558 separate determinations made up of 156 pH readings, 163 estimations of free acid, 163 estimations of total acidity, and 76 determinations of excess neutralizing ability of the duodenal contents.

## RESULTS

*Acidity in pH Units (Fig. 1).—Stomach.* The average gastric acidity in terms of pH in fasting normal subjects (pH 3.51) was less than that observed after an Ewald meal (pH 2.78),<sup>8</sup> even though the range of the individual pH values in both the fasting and digestive states was about equal. It is interesting to note that even the average gastric acidity expressed in pH units of those subjects who had some free acid in their fasting stomach contents (pH 2.37) was less than that in fasting normal dogs (pH 1.90).<sup>7</sup>

*Duodenum.* The efficient neutralizing ability of the contents of the duodenal bulb during fasting was evidenced by the constant maintenance of a difference in pH in the samples collected at the same moment from areas just above and just below the pylorus. Throughout the period of observation in the fasting state this difference averaged 2.09 pH units. Further evidence of the effectiveness of duodenal neutralization in the fasting state was found in the relative infrequency of occurrence of free acid in the duodenum. Under fasting conditions only 26 per cent of all the samples had a pH less than 3.5, the critical value we had adopted for free acid,<sup>6</sup> compared with 31.7 per cent during the observation period following an Ewald meal.

A peculiar feature, which was seen in the entire group as well as in those members of this group who were able to secrete free acid, was a progressive elevation in the average duodenal pH during the period of observation. As a result, the character of the line representing the plotted values for average duodenal pH was distinctly different in pattern from the corresponding one for average gastric pH.

The over-all average duodenal pH was 5.60, which was about equal to that we found in fasting normal dogs (5.56),<sup>7</sup> despite a much greater acidity in terms of pH in the stomach of dogs as compared with men. The much more efficient duodenal bulb neutralization in fasting dogs was also seen in the fact that only 17.2 per cent of the duodenal samples in these animals showed free acid (a pH less than the critical value of 3.5).

*Free Acid (Fig. 2).—Stomach.* The average gastric free acid in fasting normal subjects was 15 clinical units. This was less than that after an Ewald

meal<sup>s</sup> (21 clinical units), and the maximal values attained were not as great. The average gastric free acid was also much less than that in fasting normal dogs.<sup>7</sup> There was a contrast between the configuration of the line representing the plotted values of average gastric free acid and that representing average duodenal pH (Fig. 1).

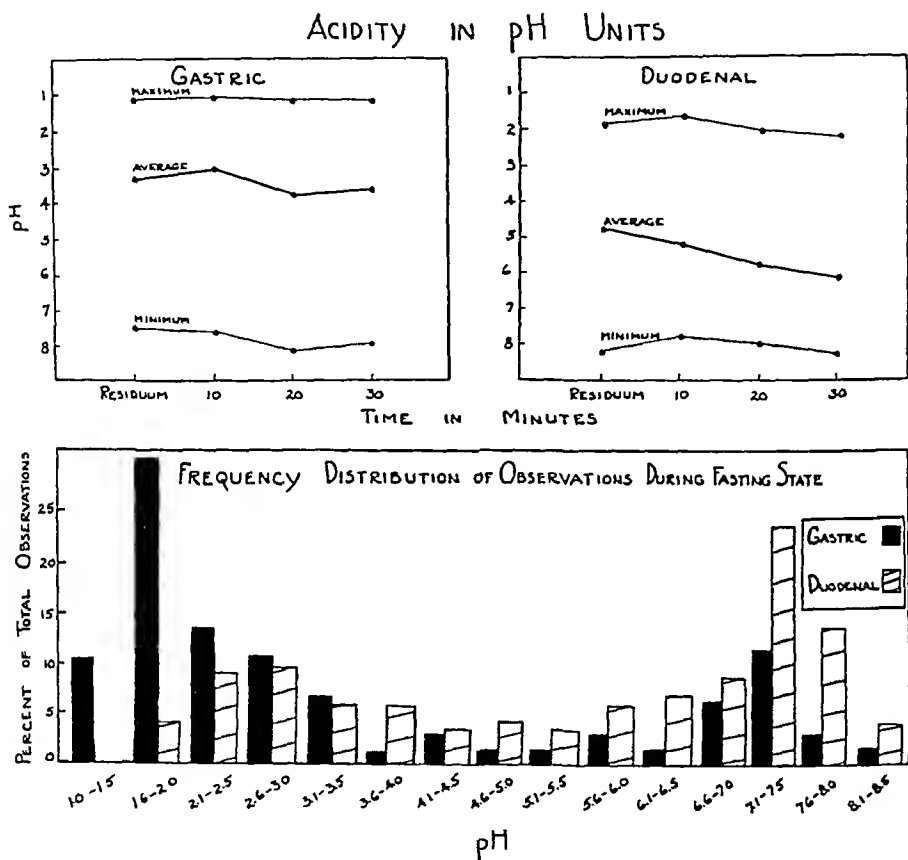


Fig. 1.—Acidity in pH units of samples collected simultaneously from just above and just below the pylorus in fasting normal people.

**Duodenum.** In the vast majority of instances the contents of the duodenal bulb under fasting conditions showed an absence of free acid; only 10 per cent of all the specimens yielded a colorimetric reaction with Töpfer's reagent, such as was considered positive for free acid. A greater percentage of these samples (26 per cent), it will be recalled, were thought to contain free acid as judged by their pH values (Fig. 1). The apparent discrepancy between these percentages may be attributed to our method of filtration and dilution preparatory to colorimetric titration which results in a number of false negative readings.<sup>6</sup> Our end point with Töpfer's reagent was about pH 3.5,<sup>6</sup> so that a more accurate figure is the one based upon the assumption that all specimens whose pH in the unfiltered, undiluted state is 3.5 or less contain free acid.

The percentage of duodenal samples positive for free acid was less than in normal people after an Ewald meal in whom free acid was found col-



orimetrically in 15.2 per cent, and electrometrically in 31.7 per cent of the samples.<sup>5</sup> An added contrast indicating the very effective neutralizing power of the contents of the duodenal bulb during fasting was the smaller percentage of subjects who had free acid in the contents of their bulb at some time during the period of observation. The comparative values are: fasting, 22.7 per cent of the subjects (colorimetrically), 40.9 per cent (electrometrically); Ewald

## FREE ACID

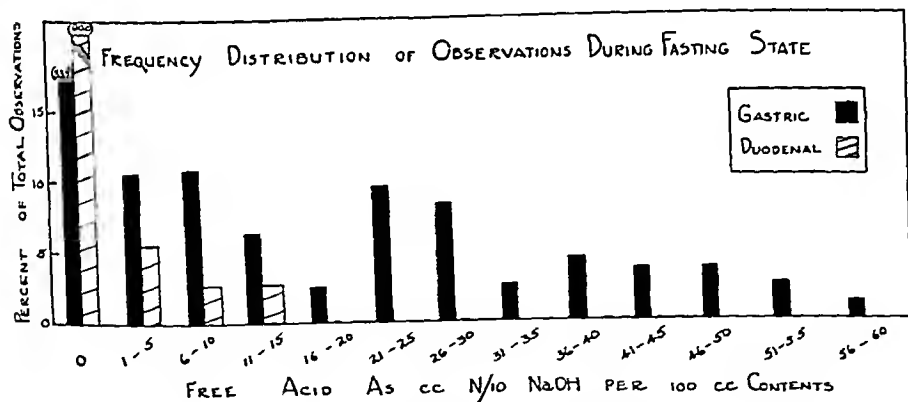
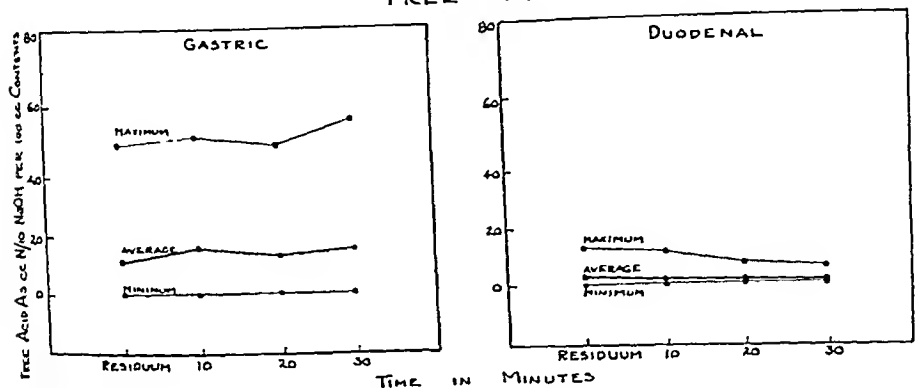


Fig. 2.—Free acid as determined on samples collected simultaneously from just above and just below the pylorus in fasting normal people.

meal, 66.6 per cent of the subjects (colorimetrically), 75 per cent (electrometrically). On the other hand, duodenal neutralization in fasting normal persons was less effective than in normal fasting dogs; despite a greater gastric acidity in the latter animals; colorimetrically, only 6.3 per cent, and electrometrically, only 17.2 per cent of the duodenal samples were positive for free acid in fasting dogs.

What little free acid was contained in the duodenal bulb contents during fasting showed the same tendency toward a diminution in average values with each successive aspiration, as had been noted in the case of duodenal pH (Fig. 1).

**Total Acidity (Fig. 3).—Stomach.** Average gastric total acidity, as with average gastric free acid, was distinctly less than that in normal people following an Ewald meal.<sup>5</sup> It was also less than in normal fasting dogs.<sup>7</sup> A differ-

ence in configuration of the graphic curves of average gastric total acidity and average duodenal pH was evident (Fig. 1).

*Duodenum.* The average value for duodenal total acidity was 18 clinical units. This and the narrower range of distribution of the individual values under fasting conditions indicate a neutralizing capacity at least equal to that observed in normal people after an Ewald meal.<sup>8</sup> The average value and the range of values, however, were not much less than those in fasting normal dogs in spite of the greater gastric acidity in the latter.<sup>7</sup>

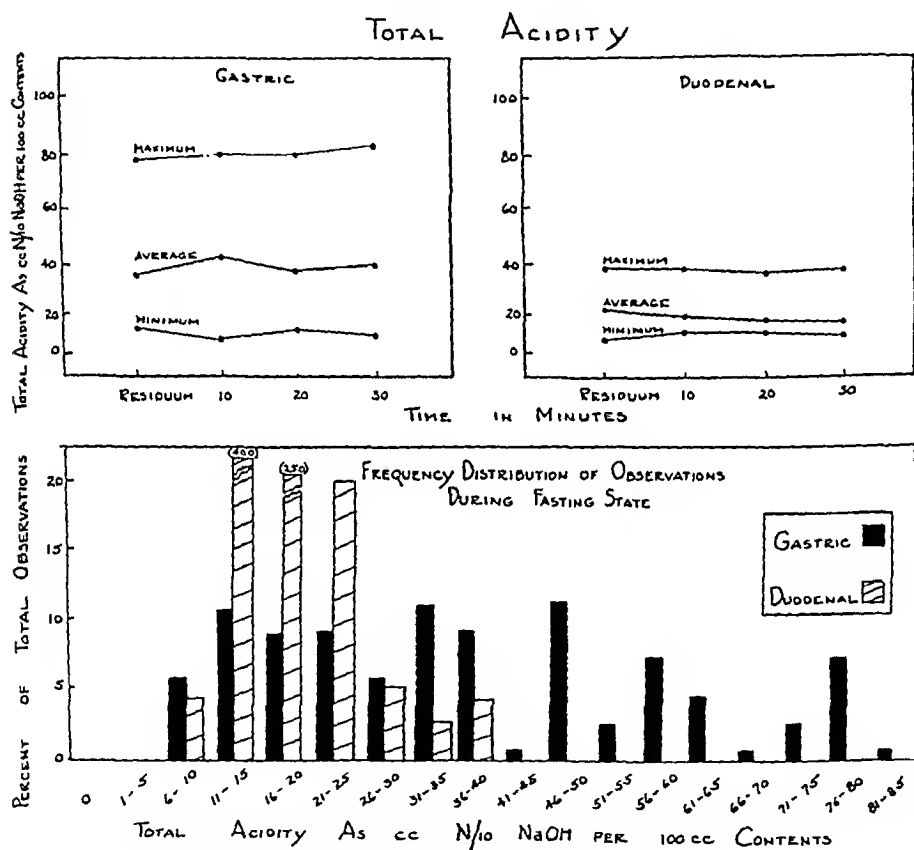


Fig. 3.—Total acidity of samples collected simultaneously from just above and just below the pylorus in fasting normal people.

Here again the contents of the duodenal bulb during fasting displayed the rather characteristic trend toward progressive decrease in acidity values. The line representing the plotted values for average duodenal total acidity assumed a form which was quite dissimilar in pattern from the corresponding curve for average gastric total acidity.

*Neutralizing Ability of the Duodenal Contents (Fig. 4).—*We previously defined excess neutralizing ability as a measure of the reserve capacity which the contents of the first part of the duodenum possess to neutralize, buffer, and dilute the secretions received from the stomach above that necessary to offset the free acid content.<sup>4, 6</sup>

The contents of the duodenal bulb in the fasting state slightly surpassed those in the digestive state, as measured by this index of neutralizing capacity. The over-all average was 17 clinical units, which was a little higher than after an Ewald meal (14 clinical units).<sup>5</sup> Only 14.5 per cent of all the samples had no excess neutralizing ability, as compared with 20 per cent of those after an Ewald meal. As measured by the same index, however, the neutralizing capacity was inferior to that in fasting normal dogs;<sup>7</sup> in the latter the over-all average value was greater (28 clinical units), the range of distribution of the individual values was wider, and the percentage of specimens negative, for excess neutralizing ability was less (8 per cent).

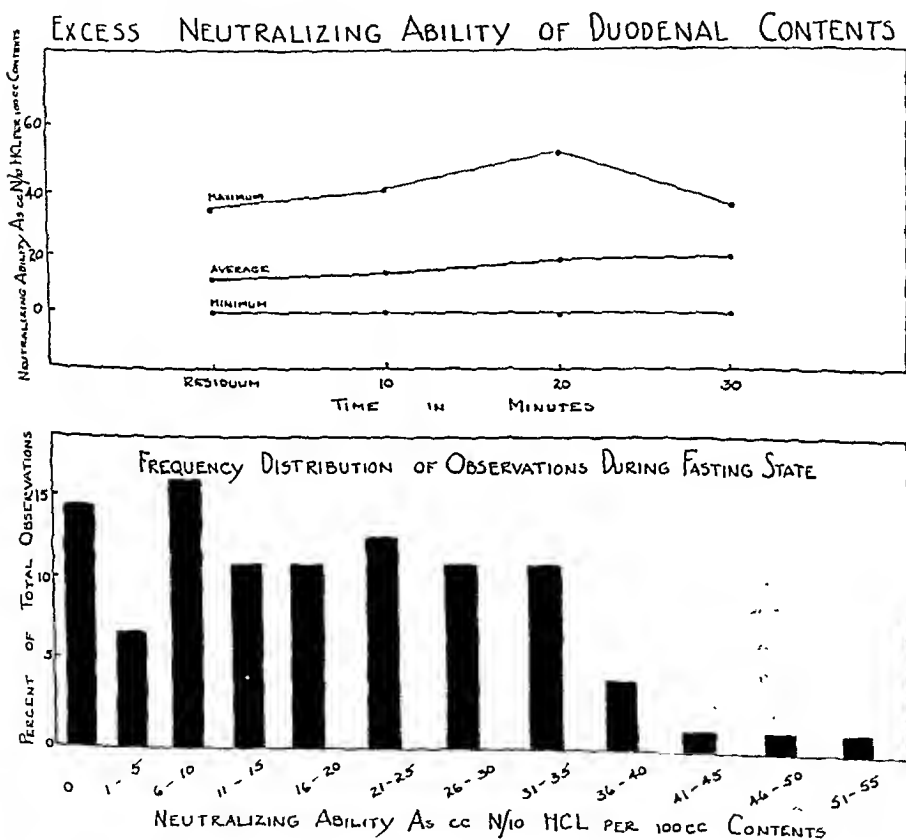


FIG. 4.—Excess neutralizing ability of the duodenal contents in fasting normal people.

The fairly typical trend toward a progressive decrease in acidity in the duodenal bulb with each succeeding aspiration was seen here in the slight but definite increase in values for average excess neutralizing ability.

#### DISCUSSION

The figures we obtained in the course of this study are not submitted with the thought that they establish an ultimate standard for the acidity in the duodenal bulb in all fasting normal people. Despite our efforts to control the origin of the specimens, undoubtedly some of them *must* have been drawn from portions of the stomach and duodenum other than those which were designated.

The existence of a true fasting gastric secretion in men is still a moot question.<sup>1, 9, 11, 23, 31-33</sup> Even though we made an attempt to avoid all extraneous influences and to secure a true resting state, it is unlikely that all normal and abnormal stimuli sufficient to influence gastric secretion were thoroughly excluded.<sup>1</sup> The values for fasting gastric acidity which we obtained, while in agreement with those observed by others with respect to both pH<sup>10, 16, 21, 22</sup> and titrable acidity,<sup>2, 3, 17, 18, 32, 33, 34</sup> are not advanced as necessarily characteristic of the normal fasting stomach. The findings, however, are useful for comparison with like values simultaneously obtained in the duodenal bulb or with corresponding values obtained under similar experimental conditions during the course of digestion.

Under our experimental conditions the reaction in the first part of the duodenum in man is acid during the fasting state, just as it is during the digestive state. In a thirty-minute observation period the contents of the duodenal bulb displayed a more or less characteristic tendency toward a progressive decrease in average acidity values, and a concomitant progressive increase in neutralizing ability. This feature was all the more interesting because it occurred independently of any similar trend in gastric acidity.

The contents of the "ulcer-bearing" duodenal bulb in fasting normal people are able to neutralize, buffer, and dilute the gastric secretions as well, and possibly even better, than during the course of digestion of an Ewald meal. A difference in reaction is constantly maintained between the adjacent pars pylorica and duodenal bulb which about equals that during digestion. While neutralization is generally effective (measured by the ability to maintain a pH of 3.5 or above), free acid is by no means constantly or consistently absent. Therefore, as in the digestive state so in the fasting state as well, the occasional presence of free acid in the contents of the duodenal bulb cannot be considered as greatly significant from the diagnostic standpoint.<sup>12, 14, 15, 19, 26</sup>

The frequently noted lack of sharp parallelism between the several indexes of gastric acidity on the one hand, and the duodenal pH as well as the corresponding indexes of duodenal acidity on the other, permits the conclusion that there is no constant relationship between the gastric acidity and that present at the same time in the duodenal bulb.

The fact that neutralization of gastric acid in the duodenal bulb is apparently more efficient in dogs than in man may be related to the fact that dogs rarely, if ever, display a spontaneous duodenal ulcer, whereas, man is very frequently afflicted with this disturbance.

#### SUMMARY AND CONCLUSIONS

1. The "ulcer-bearing" first portion of the duodenum in fasting normal people is an acid area with an average pH of 5.60.
2. The duodenal bulb in fasting normal people is able to neutralize, buffer, and dilute the gastric secretions with an efficiency which equals and possibly exceeds that during digestion of an Ewald meal.
3. Free acid is generally, but not consistently, absent in the contents of the duodenal bulb in fasting normal persons; its presence cannot be taken as a necessarily abnormal finding.

4. There is no constant relationship maintained between the acidity simultaneously determined in the stomach and in the duodenal bulb.

5. Fasting normal dogs display a greater neutralizing power in the duodenal bulb than do fasting normal men.

We wish to express our thanks to Dr. B. B. Vincent Lyon for his kind permission to use the facilities of the Gastro-Intestinal Clinic, and to Drs. Melvin Dillman and Karl Kornblum for their cooperation in performing the x-ray studies on each subject.

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## ACUTE POSTOPERATIVE LUNG ABSCESS

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### REPORT OF A CASE WITH UNUSUAL BACTERIOLOGIC FINDINGS

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ELIZABETH, N. J.

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**I**NFECTION is one of the prerequisites of the breakdown of lung tissue. The infecting organism in some cases is very specific, as in the case of a tubercular cavity or an amebic abscess of the lung. These are usually not considered under the heading of lung abscesses; however, the primary acute putrid or acute nonputrid abscesses of the lung are those that are grouped under this name.

There are observers of great experience in this field, among them Neuhof,<sup>1</sup> who readily ascribe all nonputrid abscesses to aerobic bacteria, and all putrid abscesses to anaerobic ones. Nevertheless, papers by Bueher,<sup>2</sup> Cohen,<sup>3</sup> and Varney,<sup>4</sup> with extensive bacteriologic studies, cast some doubt on the truth of this simple classification. Cohen<sup>3</sup> reported the results of an exhaustive bacteriologic study of 16 cases; he emphasized that it is highly debatable whether anaerobes are the determinant feature of putrid lung abscess. He showed that gamma streptococcus and diphtheroids were present in each of the 16 examined cases, and *Bacillus melaninogenicum* was observed in 15 of these. A great variety of other bacteria occurred in varying numbers. The findings of Bueher,<sup>2</sup> in a larger but seemingly not so meticulously studied group, indicated a total incidence of 451 bacteria in 118 cases of lung abscess. Three streptococcus groups described as hemolytic, nonhemolytic, and *viridans*, occurred 93 times. The next in frequency was the *Micrococcus catarrhalis*, occurring 61 times; diphtheroids, on the other hand, were found only 29 times, and the *Bacillus melaninogenicum* is not mentioned in his report. Varney<sup>4</sup> reported 27 cases in which certain rigid requirements were adhered to in order to obtain

all, but no other organisms than those actually present in the abscess. He stressed the point that anaerobes are found mostly in granules occurring in pus of abscesses. In two instances out of a total of 27 all the growth was anaerobic; in the other 25 there were both aerobes and anaerobes present.

These reports bring out the fact that usually more than one bacterial species is found in a lung abscess and also, that in many cases aerobes and anaerobes occur, irrespective of whether the abscess is putrid or not. A lung abscess harboring only one species of bacterium seems to be rather rare. It is of interest, therefore, to report a case of acute, nonputrid postoperative lung abscess in which two cultures were obtained from the pus aspirated from the lung. The pus was obtained before perforation into the bronchus, or operative drainage would have taken place. Both cultures showed that the only bacterium present was a micro-aerophilic, nonhemolytic, gram-positive streptococcus. The case also presented other interesting features.

G. E., female, married, 24 years of age, came under observation in November, 1940. Two years prior to this date she underwent an appendectomy for right lower quadrant pain; following this operation, the pain persisted and became her main complaint.

She had one child and had had no miscarriages. Her menstrual periods came every 35 days and were of seven days' duration; usually the pain in the right lower quadrant became more intense during the period. She had a "streptococci" sore throat in 1939, and as later verified from the physician in charge, this diagnosis was made on clinical bases alone. Her weight was 102 pounds.

On examination the essential findings were as follows: wide diastasis of the recti muscles; small mass on the right side of the pelvis, thought to be due to salpingo-oophoritis, and cervical erosion. Treatment consisted of cauterization of the cervix, followed by biweekly diathermy and aolan injections. This regime was maintained until Jan. 17, 1941. There was no appreciable relief. The patient had an upper respiratory infection from which she apparently recovered clinically.

She was admitted to the Newark Beth Israel Hospital on Feb. 9, 1941, and was operated on, under general anesthesia, the following day. The operation consisted of right salpingo-oophorectomy, resection of the presacral nerve, and reconstruction of the abdominal wall. She stood the procedure well.

On the fourth postoperative day a nonproductive, spastic, and persistent cough developed, the temperature rose to 102° F., but the physical findings were essentially negative. An expectorant was prescribed. The cough continued, became loose and annoyingly persistent. The maximum temperature on the eighth and ninth postoperative days did not exceed 100° F., and the patient was more or less comfortable. On the eleventh postoperative day, however, the cough reappeared, the temperature rose again, and the patient vomited most of her food. The temperature reached 104° F. on the fifteenth day, and dullness, but no râles, developed at the base of the right lung. Sulfathiazole was given by mouth, but the patient did not retain it. On the following day sodium sulfapyridine was given intravenously. The report of the x-ray examination of the chest was: "Pneumonic type infiltration of mesial portion of right base." The patient felt pain at a well-circumscribed point at the base of the right lung; she was tapped here, and about 1 c.c. of heavy pus was obtained; it did not seem to contain any granules. On the following two days the patient was slightly more comfortable, the cough being nonproductive and persistent. The patient was tapped again on the nineteenth postoperative day at the point of greatest pain, and a few cubic centimeters of heavy creamy pus were obtained. The bacteriologic report on the smear and the culture of the pus both times was: gram-positive, micro-aerophilic, nonhemolytic streptococcus. The following day the patient looked cyanotic and toxic, coughed, and vomited continuously, and her temperature remained above 102° F.

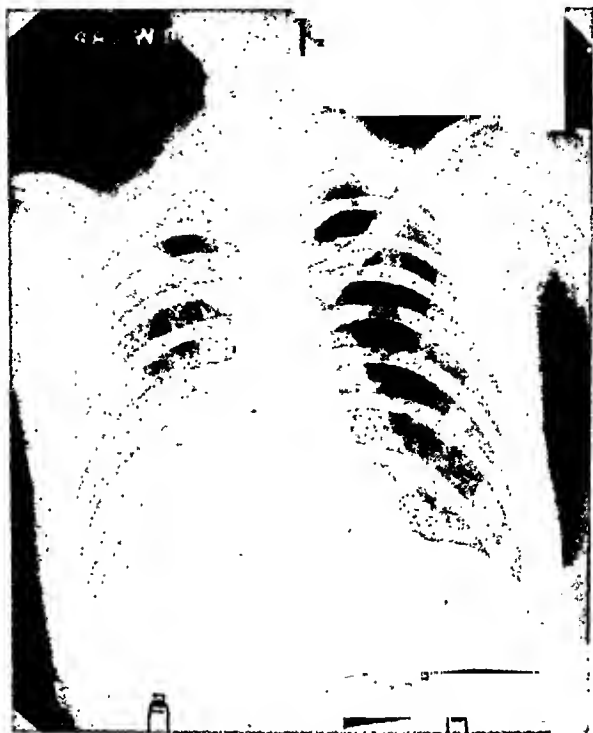


Fig. 1.—Roentgenogram taken on Feb. 26, 1941, showing pneumonic type infiltration of right lower portion of the lung.



Fig. 2.—Roentgenogram taken on March 3, 1941, showing a marked spreading of process involving more than two-thirds of right lung area. Note seat of resection shown by arrow.



In view of these alarming symptoms coupled with the physical findings, and of having obtained pus twice by tap, it was felt that a diagnosis of empyema was justified. Dr. Aaron E. Parsonnet saw the patient in consultation. He concurred in this opinion and advised immediate thoracotomy. The patient was transfused and operated upon on the same day. A two-inch part of the ninth and tenth ribs was resected in the scapular line, and the pleural cavity was opened. It was found free of fluid or inflammatory changes; there were no adhesions between the two pleural leaves. The visceral pleura was yellowish and discolored over an area of about an inch. The lung was aspirated at that point, and a few cubic centimeters of creamy pus were obtained. A part of the lung the size of a silver dollar was sutured to the parietal pleura, and iodoform packing was placed in the wound. On the next day the lung was entered at the exposed part by cautery, one finger was inserted, and friable tissue was broken up. The cavity was drained by rubber tube and packed with activated zinc peroxide cream and gauze. None of the pus specimens nor the wound afterwards had a foul odor.



Fig. 3.—Roentgenogram taken one week later, March 10, 1941, showing marked recession of the process.

Within a few hours after pneumonotomy, an impressive change occurred. The general appearance of the patient improved strikingly, the cyanosis and toxicity lessened; never returning to the same degree during the eventful and rather stormy postoperative course.

The appearance of total empyema was feared; it did not occur. At the first change of the dressing, one day after the pneumonotomy, activated zinc peroxide cream (Z.P.O.) was injected through the drain tube into the lung cavity. It resulted in such an exasperating coughing spell, that its use had to be temporarily discontinued, and powdered sulfanilamide substituted for a while. Later Z.P.O. was used again. From the very first day of pneumonotomy a bronchial fistula was present. The temperature throughout the twenty days of hospitalization following pneumonotomy continued to be about 103° F. Jaundice was noticed on the ninth day postoperatively (bilirubin was 2.7 per 100 c.c.); it subsided as the general condition improved. The cough persisted; it was spastic, with very little sputum, and was often followed by vomiting. About two weeks after pneumonotomy, burrowing appeared at the



Fig. 4.—Roentgenogram taken on March 10, 1941. Note marked improvement and aeration of lung.

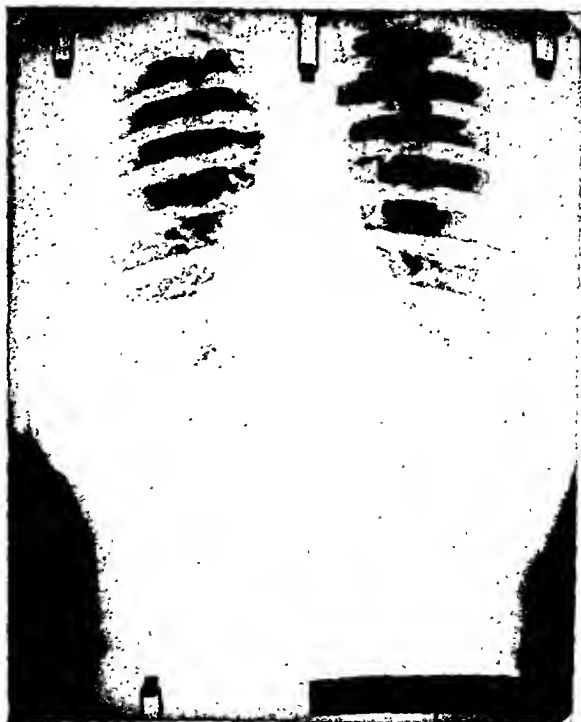


Fig. 5.—Roentgenogram of final plate on Sept. 9, 1941. Condition practically restored to normal.

upper and inner wound margins. Though the patient was cheerful, the persistent cough, the vomiting, and the high temperature caused further decline. The condition became stationary, and the patient was discharged on March 23, 1941, the forty-first day of hospitalization.

Up to April 15, the burrowing at the inner wound edge persisted, causing further loss of tissue. For a while Z.P.O. was used, followed later by azoehloramid in triacetin. On April 13, the overhanging wound edge seemed to melt away at one point; it seemed that some superimposed infection helped to destroy it. By April 15 this melting-down process neatly scooped off all the overhanging edge. From this point on the patient's condition improved rapidly; the daily temperature curve, ranging between  $102^{\circ}$  and  $102.6^{\circ}$  F. daily, tapered down, and the vomiting ceased. Her weight, which came down to the dangerous level of 72 pounds, started to come back. The recovery became uneventful. The skin wound and the bronchial fistula closed completely by May 15.

Examination on September 9 found the patient weighing  $114\frac{1}{4}$  pounds, and with no clinical symptoms. Of further interest is the fact that the patient was now completely relieved from right lower quadrant pain.

#### COMMENT

1. In this case the x-ray examination did not disclose an abscess. Jackson and Judd<sup>5</sup> reported that an abscess can be demonstrated only after drainage of some kind has been established, thus permitting the access of air into the abscess cavity. The alternative possibility of gas in the abscess would be gas formation by bacteria; such an occurrence, however, does not seem to have been reported in the literature. Palugyai<sup>6</sup> stated that "closed forms of abscess cannot be differentiated from tumor." Touroff and Neuhof<sup>7</sup> stated that acute abscess may show either cavity or dense infiltration. In this case, x-ray findings indicated a pneumonie type of infiltration.

2. There were no adhesions between the parietal and visceral leaves of the pleura, though a definite abscess was already present. This refutes the claim made by some that adhesions are always present. The abscess was not near an interlobar fissure, thus excluding the presence of invisible adhesions.

3. Pus was obtained twice, and later the abscess was found at the site of the most intense pain. This fact may be used as a lead in future cases.

4. The improvement within a few hours after draining the abscess was striking.

5. During recovery it was noted that the intercostal spaces were narrowed and that the right half of the chest, harboring the abscess originally, became smaller than the other half. Following complete closure of the wound, the chest re-expanded and the intercostal spaces widened.

6. Pus for culture is rarely obtained prior to the abscess opening into the bronchus. It cannot be obtained by bronchoscopy because the abscess did not perforate. It can hardly be obtained by operation because the examination of an abscess before perforation fails to disclose a cavity, and therefore, an operation is usually not performed. This, in large measure, is also the reason why aspiration is not attempted, the other one being the likelihood of a resultant empyema, following the needling of an abscess at an early stage. Superimposed infections easily occur after the abscess perforates, and therefore, examination will naturally disclose more than one bacterium. In the case reported, an aspiration was done because the physical findings indicated a pleural process. Both through error and lucky coincidence the needle entered the lung. Pus was found

twice, yet the pleural cavity was not infected. Thus the pus of a lung abscess was obtained under sterile conditions before the usual perforation would have occurred. The chief reason for only one bacterium being present may be due to the fact that there was complete absence of superimposed infection. It seems fair to assume that this bacterium caused the infection because the identical organism was obtained both times, and no other bacteria were found in either instance. The bacterium is a nonhemolytic or gamma streptococcus, belonging to the group that was reported by Cohen,<sup>3</sup> in each of his 16 cases. This bacterium, though micro-aerophilic, differs from the streptococci described by Meleney; his being a hemolytic streptococcus not causing any persistent fever. Meleney and Harvey<sup>8</sup> stated that "there is usually a moderate fever and a moderate pain in the wound. At times the fever may reach 103° F. for a period of days, but usually it remains at a lower level." No cultures were made after pneumonotomy. It seems that this bacterium caused burrowing, high fever, and toxicity, while even the smallest amount of burrowing persisted, though secondary infection might have played a part in this. These features make this bacterium different from the Meleney streptococci. At least one author, Reicher,<sup>9</sup> called attention to a streptococcus that was not hemolytic, but burrowing.

7. Finally, this streptococcus was the sole bacteriologic cause of a lung abscess.

#### SUMMARY

A case of lung abscess is reported. Pus for bacteriologic examination was obtained before the abscess perforated. The bacteriologic examination showed that only one bacterium was present, a micro-aerophilic, nonhemolytic, gram-positive streptococcus.

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## BENZENE POISONING IN INDUSTRY

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AS THE national defense program alters industry to suit its needs, it is placing increasing burdens on the industrial physician. In the rubber industry one of the problems confronting the medical staff is the increasing use of benzol and the physical hazards it presents.

Pure benzene,  $C_6H_6$ , is a coal tar distillate. It is a colorless limpid liquid with a characteristic odor which is rather pleasant. It boils at  $80.2^\circ C$ . Its specific gravity is 0.879. It is soluble, 0.07 Gm. in 100 c.c. of water. It is somewhat soluble in alcohol. Pure benzene is not used in industry. The commercial varieties contain from 2 to 10 per cent toluene, xylene, olefins, paraffin, and carbon disulfide. It is an excellent solvent for rubber gums, resins, celluloid, and fats of all kinds. It is the best solvent for certain types of synthetic rubber because it dries faster and is cheaper. It is these properties that make its use so prevalent in the rubber industry today.

It is used extensively in the rubber industry, in the leather industry, in varnish and paint removing, in gilding and bronzing, in spray gun paints, coal tar paints, and to a small extent as a motor car fuel.

Benzol poisoning may result from absorption of the benzene by the respiratory tract or the alimentary tract, or through the skin. In the majority of cases absorption through the respiratory tract is the common mode of entrance. Absorption through the skin is slow. A considerable amount of fumes may act as an asphyxiation agent. With the use of animals the United States Public Health Service states that concentrations as low as 5 parts per million may cause symptoms. Cases of poisoning in human beings have been found with concentrations as low as 25 parts per million. Concentrations of 50 to 100 parts per million are considered to be safe for the average person. Individual susceptibility varies. Some are able to withstand much higher concentrations than others. This factor in itself causes a big problem in industry, since ventilation adequate to maintain a concentration within safe limits is still not enough to prevent the person whose tolerance is low from absorbing the chemical.

The following statistics are those of a factory in the rubber industry that has been using benzol in small quantities for years. With the advent of large war orders and the increasing use of synthetic rubber, patients came to the company hospital with complaints of malaise, nausea, and vomiting, and in a few cases, of bleeding. An immediate check was made on all employees using benzol to any extent. Complete blood counts were done on 1,104 people. Of this number 83, or 7.5 per cent, showed mild blood changes and gave symptoms of having some slight absorption of benzol fumes. Twenty-five, or 2.2 per cent, showed severe blood changes with symptoms of severe benzol intoxication. These patients had to be treated over a long period of time. Of the above 25 cases, 9 were confined to the hospital for a period of time and received blood transfusions

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varying in number from a low of 2 to a high of 50. Three patients of the above 9 died. For absolute certainty in the diagnosis a bone marrow biopsy and study were done on all the severe cases. This degree of fatality of three patients out of 25 with aplastic anemia, or 12 per cent, is low. This low incidence was believed to be due to the extreme amount of treatment received. The treatment given will be explained later.

#### SYMPTOMS

Individual susceptibility is the governing factor in the appearance of symptoms. Some patients can apparently be exposed to large concentrations of benzol without effect. Others show symptoms when the concentration is as low as 5 parts per million. The United States Public Health Service states that young intelligent girls are the most susceptible.

Acute benzol poisoning is rare. The symptoms of an acute case are referable to the central nervous system. There are muscular tremors, salivation, violent twitchings, exhaustion, paralysis, narcosis, convulsions, and death from paralysis of the respiratory center.

The common cases are those of long exposure to fairly small concentrations of the fumes; that is, concentrations usually below 100 parts per million, with occasional sharp exposures of 500 to 1,000 parts per million, such as occur when fumes are obtained from the opening of a can of benzol or from the heating of material containing benzol. The concentration of fumes to which the patients studied were exposed varied between 50 and 500 parts per million, with the average concentration being about 100 parts per million. Then the symptoms are lassitude, malaise, nausea, vomiting, dizziness, and headaches, usually frontal in character. If there is any skin absorption, a macular dermatitis will appear at the site of exposure. As exposure continues the above symptoms become more severe. Petechiae, scattered over the body, appear. Hemorrhages under the skin and from the body cavities may occur. In one case ecchymoses and petechiae were the first symptoms that appeared. At the time of appearance the count was normal. In five days the white blood cell count was 500. Diplopia, especially for close distances, occurs in some cases. If the person is highly susceptible, or if the exposure has been great and prolonged, he may get physiologic or functional depression of the bone marrow. This varies in degree from a slight hematopoietic suppression to a total bone marrow aplasia.

The symptoms of a total aplasia are those of benzol absorption followed by prostration. On the other hand, it is sometimes strange how well the patient may appear. Aside from fatigue he may have no other complaints.

The best index of benzol absorption is the blood count. All blood counts should be done with pipettes that have been standardized by the United States Bureau of Standards. The improved Newcomer method of hemoglobin determination is preferable. All components of the blood are affected, being lowered. Most important, the total leucocyte count falls. Special reference should be made to the differential count. The ratio of polymorphonuclear cells to monocytes is changed. The number of monocytes increases and the polymorphonuclear cells decrease. The red blood cell count is lowered. If it makes a drop of 25 per cent, the employee should be removed from the fumes. The hemoglobin also falls. It has been reported by some investigators that the blood count

may be raised at first. However, when this occurs it is quickly followed by a general lowering of the count. The platelet count is lowered, dropping to practically zero in severe cases. The reticuloocyte count is also lowered. The prothrombin time is normal. The resulting picture is one of aplastic anemia. One of our patients exhibited a count of 125 white blood cells, 1,500,000 red blood cells, and 25 per cent hemoglobin. This man died. The red blood cells exhibit anisocytosis, poikilocytosis, and polychromatophilia. In all patients showing a marked blood reduction a bone marrow biopsy should be done. The degree of aplasia can be determined, and usually an accurate prognosis can be made. Patients with total aplasia always die. Patients with partial aplasia have a prognosis dependent upon the amount of regeneration present.

Urine examinations are usually negative for albumin and sugar. The urinary sulfate test, which is a ratio between the total sulfates and the inorganic sulfates present in the urine, is useful in indicating absorption of fumes. The ratio is lowered when the absorption is great. This is because the inorganic sulfates are increased. This test is useful only at time of exposure, the ratio returning to normal in about four hours when the patient is in fresh air. Normally the ratio is about 0.75. In severe exposures it may be lowered to 0.25. Lowering of the ratio is an indication for removal of the employee from exposure to the fumes.

The blood pressure may be lowered. This test is not significant unless it is accompanied by other positive findings.

#### DIFFERENTIAL DIAGNOSIS

Aplastic anemia is to be differentiated from agranulocytosis and acute leucemia. The symptoms of both of these diseases are very similar. In agranulocytosis the red blood cell and platelet counts are usually essentially normal. In acute leucemia the white blood cell count may be normal, raised, or lowered. The differential count shows a marked increase in very young white blood cells at the height of the disease of acute leucemia. The liver and spleen are usually enlarged in acute leucemia and are normal in aplastic anemia and agranulocytosis. The lymph nodes are normal in aplastic anemia and are usually enlarged in leucemia. In agranulocytosis the lymph nodes of the neck are usually enlarged and are painful and tender. Hemorrhages are common in aplastic anemia and leucemia, and are rare in agranulocytosis. Sore throat is common in leucemia and especially so in agranulocytosis. It must be recognized that the differential diagnosis between these three diseases may be difficult. Bone marrow studies usually reveal the proper diagnosis.

#### PROPHYLAXIS

This problem is one of major importance. The cooperation of the management of the company is necessary. Many different ideas have been expressed on the subject. Of course, the ideal situation would be to do away with the use of benzol. Toluene is mentioned as a substitute. This chemical, which is methyl benzene, is not as toxic as benzol because of its higher volatility. However, sufficient concentrations of the fumes of toluene will act in the same manner as benzene.

Adequate ventilation is an important prophylactic measure. A closed system of ventilation where the workman comes in contact with no fumes is ideal. Any other type of ventilation is not entirely safe. So long as any fumes are present it is possible that intoxication may occur.

The health of people working in benzol should be kept under constant supervision. Any illness or symptoms of illness should be investigated. Frequent blood counts should be made. Occasional urinary sulfate tests are of value.

The following program is suggested:

1. The testing of frequent air samples with a combustible gas indicator. Introduce ventilation in an endeavor to keep the concentration of benzol in the air as far below 100 parts per million as possible.
2. All new employees, before being permitted to work, should be given a complete blood count. Any persons with blood dyscrasias should not be permitted to work in benzol departments. No one should be allowed to work in a benzol department with a white blood cell count below 5,000, or above 13,000.
3. After ten days' employment the employee should be given a urinary sulfate test at the end of a working day. If the urinary sulfate ratio is below 50, there is a tendency to absorption, and the employee should be removed from the benzol department.
4. Any employee exhibiting symptoms should be sent to the hospital for blood studies and physical examination. A drop of 25 per cent in any blood components is sufficient reason for removal. An employee who is so removed should be given one week vacation and then placed on another job. During the week off the employee should remain as much as possible in the open air. At the end of the week the blood should be rechecked. If normal, the employee may be permitted to return to work in a nonbenzol department.

An ideal arrangement would be to use a system of repeated blood checks on exposed employees. If this were done at intervals of approximately every two weeks, it would be of benefit, but even then no positive guarantee could be given that poisoning would not occur. This type of test may be prohibitive in large industry because of the number of technicians needed to perform the counts.

#### TREATMENT

In patients with mild intoxication, with or without a drop in the blood count, the treatment is removal from the fumes and plenty of fresh air. These patients will return to normal in a few days with no medication.

In patients in whom absorption is sufficient to cause a marked change in the blood picture more active treatment is necessary. These patients are placed at absolute bed rest. Multiple small whole blood transfusions (about 250 c.c.) are given. One patient was given 50 transfusions, another 36. The patient is alkalized with sodium or potassium citrate on the possibility that this might prevent a reaction to the transfusions due to acidosis. When severe reactions occur, blood plasma may be used in place of whole blood.



Direct bone marrow transfusions may be given as often as twice weekly. In this procedure 2 to 5 c.c. of bone marrow are removed from the sternum of a compatible donor and introduced directly into the sternum of the patient. From the same donor 250 to 300 c.c. of blood are drawn and transfused into the patient. This is followed by 1,000 c.c. of normal saline solution.

Ten milligrams of liver are given intramuscularly daily. Large daily doses of liver, iron, calcium, phosphorus, yellow bone marrow, and multiple vitamins are given by mouth. A total of 400 to 600 mg. of ascorbic acid are given daily by mouth. This has been claimed by some investigators to be a necessary adjunct in treatment. A full diet is prescribed. The patient's general hygiene is improved as much as possible. Bleeding areas are stopped if at all possible. Pentnucleotide has been used and found to be effective in some cases.

The resistance of these patients is low due to the agranulocytosis. Secondary infection, the most common of which is a Vincent's organism infection of the mouth, is one of the causes of death. For certain types of severe secondary infection sulfadiazine in 4 Gm. daily doses has been used. Sulfanilamide powder was used on one case of secondary streptococcus infection with good effect. However, the sulfone products must be used with care since they are bone marrow depressants.

The response to therapy is slow since a considerable time is necessary for the bone marrow to regenerate. One patient has been under treatment for twelve months. He still has a low hemoglobin and red blood cell count. It has been suggested that a splenectomy be done on patients who do not respond to therapy, the rationale being that the spleen has an inhibiting effect on the bone marrow. This procedure has been reported to be successful in several patients who otherwise would probably have died.

The treatment at best is not wholly satisfactory. In too many patients the disease proves fatal. The problem of the use of benzol has become a large and vital one and as yet is not solved.

Probably the most important fact to remember about the use of benzol for industry is never to sacrifice caution in the use of benzol because the result of carelessness with this chemical is usually death.

#### SUMMARY

Out of 1,104 people tested who were exposed to concentrations of benzol fumes averaging about 100 parts per million for varying periods of time, 83, or 7.5 per cent, show slight blood changes, indicating slight absorption of the fumes. Twenty-five, or 2.2 per cent, showed marked blood changes. Nine of these 25 patients had a severe enough aplastic anemia to demand hospitalization. Three patients died, a 12 per cent fatality out of all the patients showing absorption, or 0.27 per cent fatality out of all the people exposed to the fumes. This degree of fatality of patients with aplastic anemia is considered to be low. The form of treatment was multiple whole blood and direct bone marrow transfusions as well as liver, iron, calcium, phosphorus, yellow bone marrow, and multiple vitamin therapy.

Prophylactic measures recommended are adequate ventilation and constant medical supervision, which is best done by frequent blood examinations.

## THE BLOOD PATTERN AS A CLUE TO THE DIAGNOSIS OF MALIGNANT DISEASE

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THE early diagnosis of carcinoma is a goal eagerly sought by science in order that treatment may be undertaken before it is too late. The greatest obstacle to progress in cancer therapy is the vagueness of the early complaints and the obscurity of physical findings. As Fitz remarked in 1939:<sup>1</sup>

"Cancer presents a bewildering problem. To me the impressive feature of watching such cases was the apparent suddenness of onset of important symptoms. . . . If this is true, what is badly needed is a test for carcinoma analogous to the tuberculin test for tuberculosis and the Wassermann test for syphilis, so that cancer can be recognized in its preclinical state. Until such a test is discovered, cancer inevitably must continue to be discovered in most cases, when it is already far advanced."

To this end investigators have utilized the laboratory in an effort to discover the presence of cancer cells in the body before the appearance of clinical symptoms and signs. In 1940 these statements appeared<sup>2</sup> regarding the status of the search for a serologic test for cancer:

"It may be doubted whether it will be possible for science ever to place at our disposal a test so delicate as to indicate the existence of a few cancer cells in the human body; yet this is precisely the type of test most desired."

"Ordinary examination of the blood reveals no condition peculiar to malignant disease. Most observers have found a decrease in red cells, some have found a diminution in hemoglobin, others have noticed an increase in leukocytes. All these findings, however, are present only in the later stages of the disease. In many cases reported, the notation *blood negative* will be found."

According to Stahl,<sup>3</sup> the origin of blood and the origin of cancer are very closely interwoven in primal embryologic study. Investigation indicates that there is a specific cancer cellule. This is always an immature, never a mature, cell. In the normal organism a growth-control hormone is present; when this ceases to function, the cancer cells grow, and if this growth is not checked, the neoplasm progresses, causing devastation and death.

In 1935 Boyd<sup>4</sup> stated that cancer may be regarded in two ways. It may be the expression of a power for unlimited proliferation, which develops in a tissue at a certain age in its genetic life history; or a malignant condition may be imposed on cells from without by a number of carcinogenic agents, such as coal tar or dibenzanthracene. Of the exact mechanism nothing is known, and may never be known.

Does the answer lie in a study of the blood? In a physiologic sense the blood is a part of every tissue, and any change involving the fixed tissues of the body must affect the blood. Thus we have a picture of deformity of the red blood cells in anemias sufficient to clinch the diagnosis even without clinical evidence. Hematopoiesis is the sum total of the integrated functioning of all the tissues of the body.

There is not space to review the various tests which have been proposed to detect the presence of cancer cells in the human body. Fuchs's<sup>5</sup> test for malignancy appeared in 1925. He examined the blood of 92 persons with cancer and 48 controls without malignant disease. The blood serum from every cancer patient digested heated human fibrin; the blood serum of the controls was invariably negative. This test has furnished the basis for later investigation. Recently Robinson<sup>6</sup> used a modification of the test in 200 patients in the Vanderbilt University Hospital. Rosenthal<sup>7</sup> published results with this method at the University of Pennsylvania. Esculies of Buenos Aires<sup>8</sup> discussed results in 1940.

They stated that they isolated an ether-soluble protein from the serum of cancer patients, which was either the product of abnormal metabolism, or a protective ferment called into being against the invasion of abnormally metabolized protein derivatives. Further investigation is to be carried on to determine whether the protein isolated by these authors possesses fermenting activity in the presence of the same serum from which it was extracted, as well as the precise polarimetric deviation.

This test has the following limitations:

1. The growth must be over six months old.
2. The patient should not have been operated upon or have received radium or roentgen-ray treatment for at least a month.
3. The tumor must be in a period of active growth.

Pfeiffer's<sup>9</sup> crystallization method for the diagnosis of cancer, which appeared in 1938, has also been widely used. He claimed that the patterns which crystals assumed after the addition of diluted blood to a solution of copper chloride was a method of differentiating between health and disease. A certain cross formation was characteristic of tuberculosis; a wing pattern was indicative of cancer. In cancer, however, the blood picture is decentralized and disorganized. The more numerous and defined the groupings, the more advanced the cancer. The fact that cancerous blood actually produces these differences, which are uniform in kind, is readily established even with a few tests. Rascher of Munich<sup>10</sup> and Gruner of Montreal<sup>11</sup> have used Pfeiffer's method with personal modifications and have obtained a high degree of accuracy in diagnosing cancer.

The test is fairly simple. One drop of blood from a finger prick is allowed to fall into 1 c.c. of distilled water; after thirty minutes 2 drops of this are introduced into 10 c.c. of a 20 per cent solution of copper chloride. After thirty seconds the mixture is poured out upon specially prepared plates resting in a chamber. Readings of the crystallizations are made after eighteen hours.

Gruner uses polarized light to read the slides. He secured positive results in 110 of 122 patients, and in 33 normal persons the test was positive only once. Rascher examined 246 persons. In 98 there were no signs of cancer, and the clinical picture agreed in every case. In 148 patients the plates indicated cancer. In 11 the diagnosis was incorrect, three being normal, and eight having sarcoma instead of carcinoma. In nine the clinical diagnosis of cancer was doubtful. In 128 the plates were correct; all these patients had carcinoma.

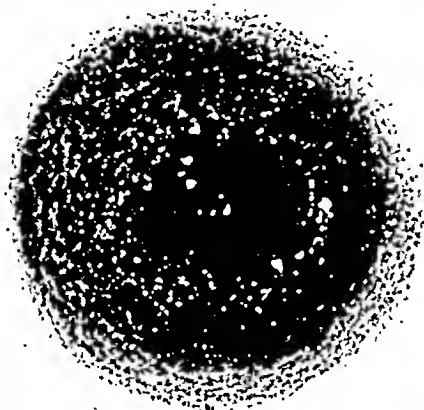


Fig. 1.

For this test the technique must be extremely exact, and only fresh blood can be used (not later than five hours old). In some instances the test was repeated after excision of the cancer. In some cases the test became negative, which was interpreted as indicating complete removal. If the test was repeatedly positive, the patient was watched carefully, even though no tumor could be located.

Reports from the Lankenau Hospital<sup>12</sup> show an 80 to 85 per cent of reliability in diagnosing early cancer. The test needs simplification and is available only in a large medical center because of the difficult technical procedures. The cancer pattern is described as two wings rising from a straight basis line, both wings extending on the same side of the basis line.

The blood sedimentation rate has been employed for many years as a diagnostic aid. Its use in gynecology, obstetrics, and urology, and the different methods of performing this test are set forth in an excellent article by Hirsh.<sup>13</sup> He outlines the Schiller modification of this test and its wider application, and analyzes the various theories regarding the factors which influence the speed with which the erythrocytes settle. The most probable explanation is that toxin production involves a breaking down of tissue proteins, and the products of disintegrated tissue proteins stimulate fibrinogen formation. The following statement of Hirsh is significant:

"We believe the sedimentation rate depends on the presence of a foreign protein in the circulation. In pregnancy, this is the protein of fetal catabolism;



Fig. 2.

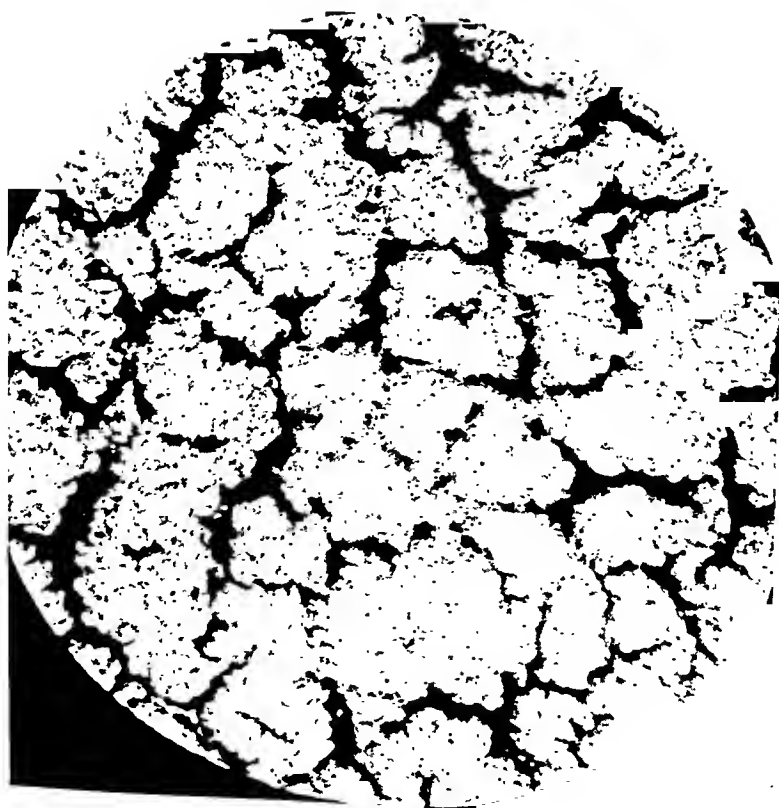


Fig. 3.

in infection, it is the protein of bacteria, and in malignancy or coronary occlusion, it is the protein of necrotic tissue."

The test, although not specific for any one condition, is of great value in estimating the degree and progress of disease. According to Schiller:

"If a malignant growth be removed operatively, the blood sedimentation rate should return to normal within six weeks if complete removal has been obtained. If the rate returns to normal and remains normal at least six months, one may give a guardedly favorable prognosis. If the rate becomes abnormal within this time, it is suggestive of local recurrence of the growth or metastasis."

Wolfson and his co-workers<sup>14</sup> recently published an article on early diagnosis of malignant metastases to the spine, and discussed the value of the erythrocyte sedimentation rate, which has a definite correlation with metastatic involvement of bone. Malignancy is often associated with an elevated phosphatase level and a rapid sedimentation rate.

In 1939 Goldberger<sup>15</sup> published his report of a rapid bedside test for measuring the blood sedimentation rate. He pricked the finger of the patient and allowed a small drop of blood (about 3 mm. in diameter) to touch the under-surface of a clean glass slide. Since the first drop collected will be thickly rimmed, a second and third drop are touched to the slide. Then the slide is righted and the drops of blood are allowed to clot and dry spontaneously in a horizontal position. When dry, the blood is observed macroscopically by holding the slide up to the light. The criteria for reading the film are as follows:

1. Fineness or coarseness of general detail.
2. Character of meshwork, if present.
3. Presence or absence of central agglutination.
4. Presence or absence of peripheral rings.

With these in mind, four classifications of sedimentation rate can be made:

1. Normal—very fine detail; no meshwork; gradual transition from periphery to dark central agglutinated mass.
2. Moderately rapid—detail somewhat coarse, but uniform throughout; meshwork not particularly noticeable; there may be the beginning of a central agglutinated mass.
3. Rapid—coarse detail; meshwork fine, but easily observed; no central agglutinated mass.
4. Very rapid—very coarse detail; definite meshwork; no central agglutinated mass.

By careful study of a great number of slides, Goldberger found that this method proved to be a simple, rapid, qualitative gauge in the diagnosis of coronary thrombosis, rheumatoid arthritis, salpingitis, etc. He found that the rate at which the red blood cells settle is directly proportional to the degree of their rouleau formation. Moreover, there was no correlation between the red blood count and the character of the drop of blood on the slide.

This problem of the blood sedimentation continues to give rise to much speculation. Rubin and Smith<sup>16</sup> believe that the volume of the erythrocytes



Fig. 4.

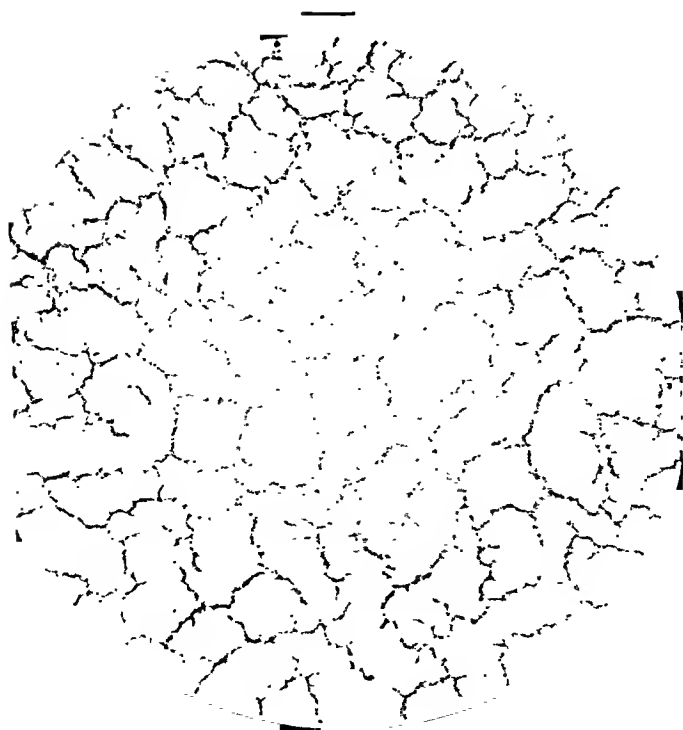


Fig. 5.

exerts an important influence on the sedimentation reaction under all conditions, and that probably physiologic changes in the sedimentation rate are also, to a great extent, influenced by the erythrocyte factor.

Davison,<sup>17</sup> in studying the separation of the blood cells from plasma (sedimentation), concludes that the process is dependent on rouleau formation. In normal or slowly sedimenting blood, each rouleau contains few erythrocytes, and the cell aggregates appear evenly distributed. In pathologic blood showing rapid sedimentation, each rouleau contains many corpuscles, and the rouleaux are clustered together, leaving large areas of free plasma between them. The sedimentation rate is directly proportional to the size of the cell aggregates or rouleaux.

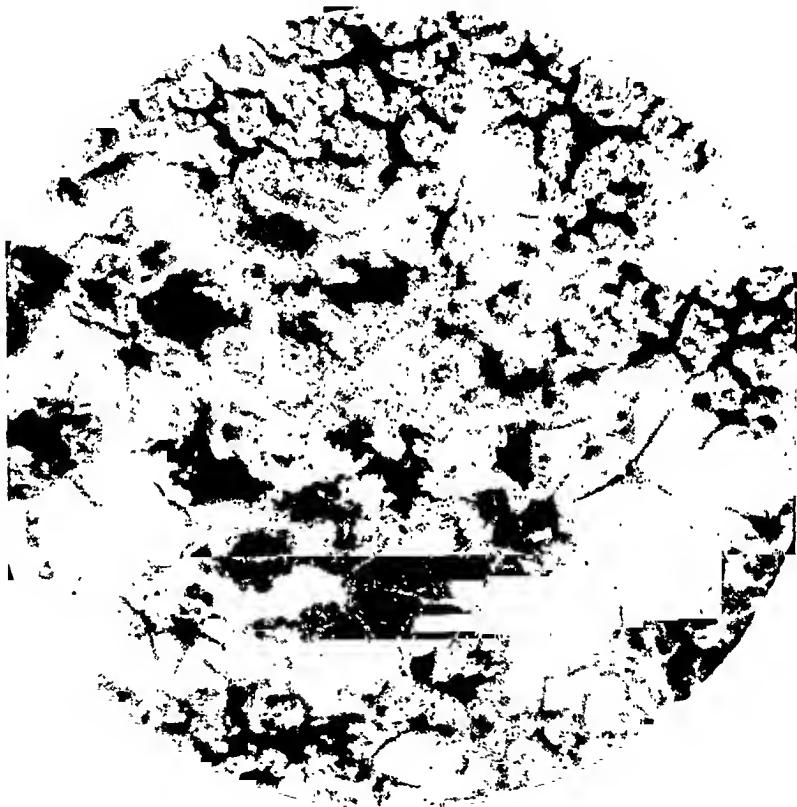


Fig. 6.

Impressed with the simplicity of Goldberger's method, the author decided to obtain a similar record of the blood on every patient, to see whether it would afford material aid in diagnosis, prognosis, and therapy. In each case, the finger was pricked, and 3 small drops of blood were collected on a glass slide and allowed to dry after the method of Goldberger. The patterns which the blood assumed, both macroscopically and microscopically, have proved of great value, especially when considered with clinical and laboratory findings.

The most interesting phenomenon was the distinctive pattern of the blood obtained from cancer patients. The pattern characteristic of normal blood



and of blood from patients with nleer, inflammatory disease, etc., is devoid of meshwork. There is no breaking up of the fibrin; the blood is more compact. In the central part of each drop of blood is a dark agglutinated mass (Fig. 1). Invariably, the pattern formed by cancerous blood is that which I describe as the "dotted curtain" type (Fig. 2). Peripheral rings are absent, and no agglutinated mass can be seen in the center. The blood is broken down into "dots." The microphotograph of normal blood (Fig. 3) and (Fig. 5) is contrasted with the microphotograph of cancerous blood (Fig. 4) and (Fig. 6). Under the microscope the cancerous blood shows numerous three-cornered, tri-asteroid spicules scattered throughout. In no other condition have these spicules or rays been found in such abundance.

In six conditions listed in Table I I have demonstrated that a positive pattern was obtained. In all these, however, the clinical findings have differentiated the condition from cancer. Moreover, the positive reaction gradually changed to negative as the proper treatment was instituted. The laboratory analysis of the blood shed no light on the problem of diagnosing cancer.

TABLE I  
CONDITIONS YIELDING TEMPORARY CARCINOMA PATTERN

|                       | POSITIVE | NEGATIVE | TOTAL |
|-----------------------|----------|----------|-------|
| Acute rheumatic fever | 3        | 7        | 10    |
| Rheumatoid arthritis  | 2        | 2        | 4     |
| Active tuberculosis   | 5        | 8        | 13    |
| Pregnancy             | 3        | 4        | 7     |
| Pernicious anemia     | 2        | 2        | 4     |
| Coronary disease      | 4        | 6        | 10    |
| Total                 | 19       | 29       | 48    |

TABLE II  
CASES OF APPARENTLY CURED CANCER

|   | POSITIVE | NEGATIVE | TOTAL |
|---|----------|----------|-------|
| Clinically Cured After Surgery:                       |          |          |       |
| Breast  | 0        | 2        | 2     |
| Stomach   | 0        | 1        | 1     |
| Rectum  | 1        | 3        | 4     |
| Prostate gland  |          | 2        | 3     |
| Clinically Cured After Irradiation or Radium Therapy: |          |          |       |
| Epithelioma, face                                     | 0        | 1        | 1     |
| Hodgkin's disease                                     | 0        | 1        | 1     |
| Breast  | 1        | 3        | 4     |
| Sarcoma   | 0        | 1        | 1     |
| Total   | 3        | 14       | 17    |

The same transition from positive to negative blood pattern on the slide occurred when "cures" were obtained in cancer patients, either by total extirpation surgically of the neoplasm, or radium implantation, or deep x-ray therapy.

The cases I have had in this category are noted in Table II.

Although the cases listed in Table II are few, the tendency is for the blood pattern to approach normal with eradication of cancerous elements. In the great majority of cancer cases, whether treated or untreated, the blood shows the

typical carcinoma pattern described above. It is so characteristic that it is usually possible to identify at a glance which of the slides bear the cancer pattern merely by holding them up to the light.

No relation has been found between the blood count and the carcinoma pattern. The similarity of the blood analyses in normal and cancer patients is apparent from a study of 50 unselected cases in each category, as seen in Table III.

TABLE III

| TYPE      | NO. OF CASES | HEMOGLOBIN | ERYTHROCYTE COUNT      | LEUCOCYTE COUNT |
|-----------|--------------|------------|------------------------|-----------------|
| Normal    | 50           | 70%-92%    | 3,200,000 to 4,500,000 | 7,500 to 10,000 |
| Carcinoma | 50           | 40%-90%    | 3,000,000 to 4,200,000 | 5,900 to 18,000 |

Tables IV and VI include analyses of the various conditions encountered in my practice since 1939, and the type of blood patterns which were noted on the slides. In some instances, these tests were made on the same patient weekly or monthly, before and after treatment for the specific condition.

TABLE IV  
GROUP OF NONCANCER CASES

| DISEASE                     | POSITIVE | NEGATIVE | TOTAL | % POSITIVE |
|-----------------------------|----------|----------|-------|------------|
| Appendicitis                | 0        | 10       | 10    | 0          |
| Diverticulitis              | 1        | 4        | 5     | 20         |
| Encephalitis                | 0        | 1        | 1     | 0          |
| Endometriosis               | 1        | 1        | 2     | 50         |
| Epilepsy                    | 0        | 6        | 6     | 0          |
| Gall bladder disease        | 0        | 28       | 28    | 0          |
| Hypertension                | 0        | 15       | 15    | 0          |
| Meningitis, tuberculous     | 0        | 2        | 2     | 0          |
| Pneumonias                  | 0        | 5        | 5     | 0          |
| Poisoning (methyl alcohol)  | 0        | 2        | 2     | 0          |
| Tuberculosis (arrested)     | 0        | 4        | 4     | 0          |
| Ulcer, gastric and duodenal | 0        | 34       | 34    | 0          |
| Ulcerative colitis          | 0        | 12       | 12    | 0          |
| Miscellaneous               | 0        | 6        | 6     | 0          |
| Total                       | 2        | 130      | 132   | 1.5        |

Thus in this group of noncancer cases selected at random, there was a positive reaction in the blood in two conditions only; the margin of error was only 1.5 per cent. All readings in cases of appendicitis were normal regardless of the pathology. A case of ruptured appendix with peritonitis, myocardial damage, hypertension, diabetes mellitus, and a high nonprotein nitrogen reading showed a negative blood pattern. There were no positive readings in the gastric and duodenal ulcer patients, even when the hemoglobin was lower than 65 per cent. In the arrested stage of tuberculosis a normal pattern was seen, although a positive pattern was obtained in several patients with active tuberculosis. Also, in cases of coronary occlusion a positive pattern was noted about the second or third day. There was a return to normal as the condition of the patients improved.

Table V contains a classified list of cases of proved cancer. The slides of 140 of these patients were reviewed. In this series the age incidence is indicated.

TABLE V

| AGE GROUP      | SEX  |        | TOTAL NUMBER OF CASES |
|----------------|------|--------|-----------------------|
|                | MALE | FEMALE |                       |
| Under 40 years | 5    | 4      | 9                     |
| 40 to 50 years | 5    | 12     | 17                    |
| 50 to 60 years | 19   | 16     | 35                    |
| 60 to 70 years | 23   | 18     | 41                    |
| Over 70 years  | 17   | 21     | 38                    |
| Total          | 69   | 71     | 140                   |

The results of the blood test for cancer are tabulated in Table VI.

TABLE VI  
PROVED CARCINOMA CASES

| SITE                        | POSITIVE | NEGATIVE | NUMBER OF CASES           |
|-----------------------------|----------|----------|---------------------------|
| Adrenal glands              | 1        | 0        | 1                         |
| Bladder                     | 3        | 1        | 4                         |
| Breast                      | 9        | 0        | 9                         |
| Breast, recurrent           | 3        | 0        | 3                         |
| Breast, metastases to lung  | 4        | 0        | 4                         |
| Breast, skeletal metastases | 6        | 0        | 6                         |
| Gastrointestinal tract:     |          |          |                           |
| Esophagus                   | 1        | 0        | 1                         |
| Stomach                     | 35       | 2        | 37                        |
| Colon                       | 27       | 0        | 27                        |
| Rectum                      | 11       | 0        | 11                        |
| Epidermoid carcinoma, cheek | 1        | 0        | 1                         |
| Epidermoid carcinoma, nose  | 1        | 0        | 1                         |
| Throat                      | 1        | 2        | 3                         |
| Larynx                      | 1        | 0        | 1                         |
| Skin                        | 1        | 0        | 1                         |
| Pancreas                    | 4        | 0        | 4                         |
| Prostate gland              | 5        | 1        | 6                         |
| Uterus                      | 7        | 2        | 9                         |
| General carcinomatosis      | 1        | 1        | 2                         |
| Hodgkin's disease           | 2        | 1        | 3                         |
| Sarcoma, osteogenic         | 0        | 2        | 2                         |
| Miscellaneous               | 4        | 0        | 4                         |
| Total                       | 128      | 12       | 140, or<br>91.4% positive |

A comparison of the noneancer and cancer cases revealed that a positive pattern was often obtained when there was no sign of anemia. The red blood cell count was an unreliable guide as to the state of the patient and the stage of the disease. With decrease in the cell count there was a corresponding decrease in the hemoglobin. A leucopenia was noted in patients with skeletal metastases. Increase in the white blood cell count invariably denoted peritoneal involvement or infection. But the red blood cell count, the white blood cell count, and the hemoglobin were of no help in differentiating between cancer and noneancer cases.

Two representative cases are herewith reported in some detail to illustrate the value of the test in diagnosis and treatment.

CASE 1.—J. C., a white male, aged 47 years, was seen at the office on January 6, 1940. At this time he stated that he had always been well until about two years before. In January, 1938, he began to have attacks of mid-epigastric pain, which occurred an hour after meals. These were relieved, at first, by alkalis or by vomiting. Attacks increased in frequency and severity. In February, 1939, he was x-rayed and told that he had a "growth" in the stomach. He was put on a diet and given "injections."

He continued on this regime for another year. Heartburn, postprandial pain, and vomiting occurred at frequent intervals, and the patient became weaker. He had a good appetite but was afraid to eat because of the subsequent pain and distress. He lost 30 pounds and began to complain of dyspnea on exertion.

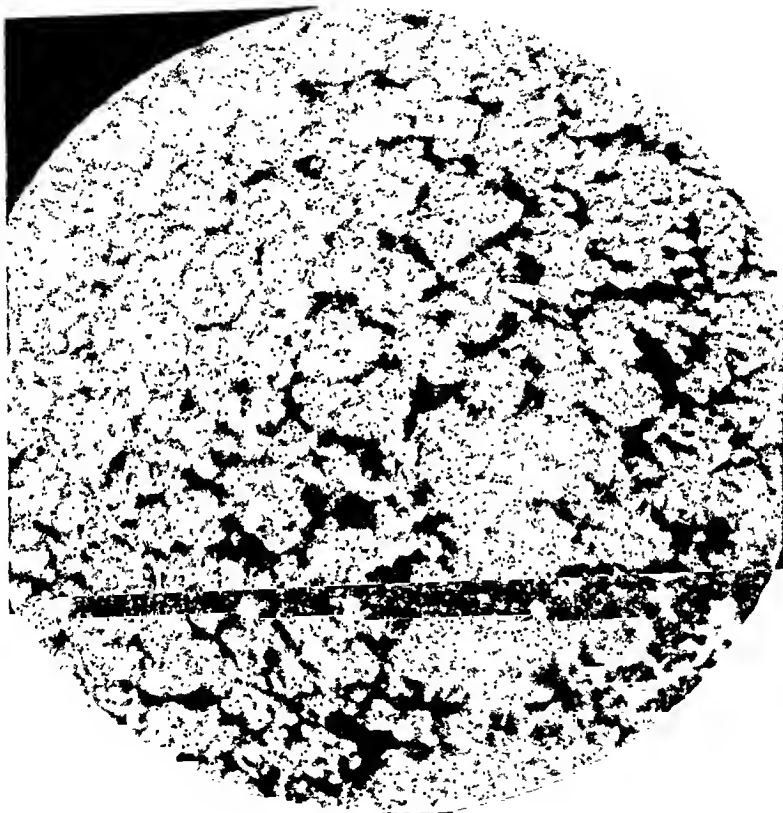


Fig. 7.

When first seen at our office in January, 1940, he stated that the pain had increased in severity. However, it never radiated to back or shoulders. He had no cough, hemoptysis, hematemesis, or melena. He was thin, pale, and listless. There was slight tenderness in the epigastric region, but no palpable mass. Three drops of blood on the slide showed a normal pattern. The patient was placed on a Sippy regimen. The following month the blood test was repeated and showed no change.

Because of inability to retain food and continual pain, the patient was seen again at the office on March 10. Another slide of the blood was made. In this the central nucleus was absent and there was definite formation of spicules. Since I felt that he had gastric cancer, patient was immediately referred to the hospital for surgery.

X-ray examination of the gastrointestinal tract was made. The impression of the radiologist was gastric ulcer, prepyloric.

Examination of the blood showed 44 per cent hemoglobin, 3,630,000 erythrocytes, and 8,400 leucocytes. The differential count was normal. There was very slight variation

in the size and shape of the red blood cells. The blood sugar was 95 mg., the nonprotein nitrogen was 33 mg. The benzidine test for occult blood in the stool was positive.

Gastric analysis on March 11, 1940, showed the following:

|                                 |         | <i>Free Acid</i> | <i>Total Acidity</i> |
|---------------------------------|---------|------------------|----------------------|
| Fasting                         | 68 c.c. | None             | 8 degrees            |
| 1st specimen                    | 3 c.c.  | 10 degrees       | 32.5 degrees         |
| 2nd specimen diluted with water |         | 10 degrees       | 16 degrees           |
| 3rd specimen                    | 10 c.c. | 20 degrees       | 80 degrees           |
| 4th specimen diluted with water |         | 12 degrees       | 29 degrees           |
|                                 |         | 52 degrees       | 165.5 degrees        |

On March 15, 1940, a subtotal gastrectomy was done for what appeared to be a prepyloric ulcer. A regional node was also excised for pathologic examination. The pathologic report, to the surprise of radiologist and surgeon, was gastric ulcer with small focus of carcinoma and metastasis to the regional lymph nodes. Fig. 8 shows a portion of the stomach with neoplasm removed at operation.

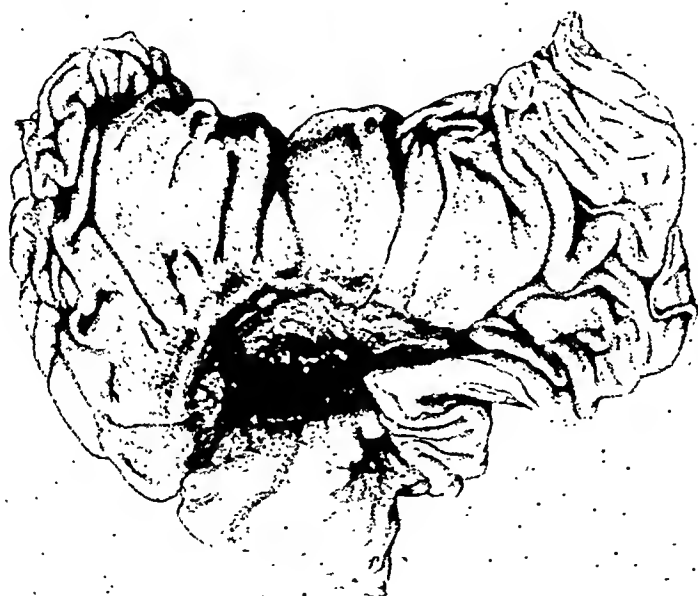


Fig. 8.

Except for an upper respiratory infection on the fifth to eighth postoperative days, the patient made a satisfactory convalescence, and was discharged improved April 7, 1940.

Six weeks after operation he was seen at the office. Examination of the blood showed that the pattern on the slide was now that of normal blood. This patient has been seen at frequent intervals since operation. There has been no recurrence of symptoms clinically. He works every day and has gained over 60 pounds. To date, January 15, 1942, the blood pattern on the slides has remained normal.

CASE 2.—D. P., a housewife, 46 years old, was admitted to the General Hospital on January 1, 1941, with the chief complaint of pain in the lower abdomen above the pubis, more severe after the menses. She stated that for the last three months menstrual pain had started about three days before the onset of flow, continued throughout the menstrual period of six or seven days, and persisted for a week after cessation. There was also a watery pinkish discharge for three days following menstruation.

She reported no previous serious illness. Catamenia began at the age of 15, a regular twenty-eight-day cycle, heavy flow, of nine days' duration. She had borne six children, all normal deliveries.

The patient's mother died of cancer of the uterus. One sister had uterine fibroids. There was no family history of tuberculosis, heart disease, allergy, or diabetes mellitus.

Physical examination revealed tenderness in both tubo-ovarian regions and in the suprapubic area. No masses or spasm were noted. The cervix was low near the introitus, large, both lips somewhat firmer than normal, both containing several small cysts. There was slight cystocele and rectocele.

Examination of the blood revealed 70 per cent hemoglobin, 4,100,000 erythrocytes, and 13,000 leucocytes. The differential count was normal. Three drops of blood on the slide, however, showed definitely the carcinoma pattern.

The clinical diagnosis was uterine fibroids.

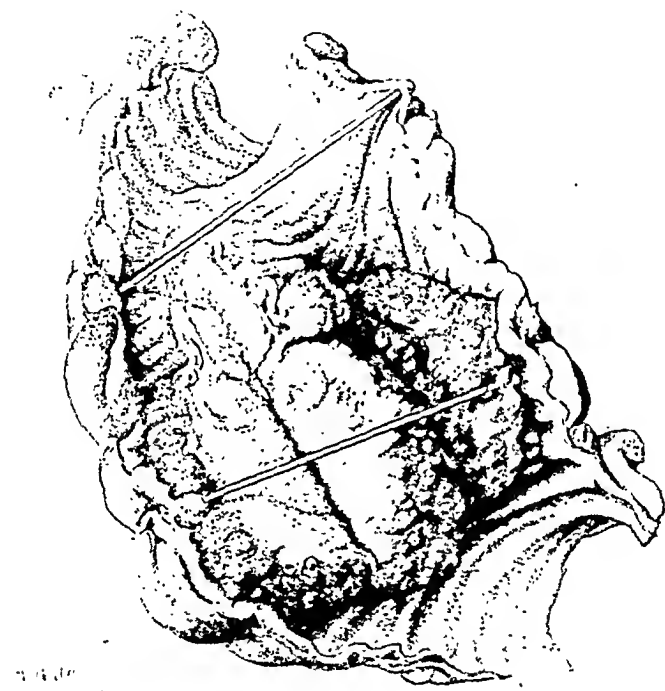


Fig. 9.

Operation was done on the following day, January 2, 1941. Laparotomy revealed a large tumor mass attached to the sigmoid. The tumor and the adjacent mass were brought out through the incision, and the wound was closed around them. On the ninth day the exteriorized loop of bowel and the tumor were removed (Fig. 9). The pathologic report was as follows:

The segment of sigmoid colon removed measured 18 by 8.5 cm. Protruding into the lumen and causing constriction, but not complete obstruction, was a large cauliflower growth, measuring 8 by 6 by 4 cm. Microscopically, the tumor mass was composed of columnar, glandular, epithelium; several mitotic figures were noted. The tumor invaded the submucosa. Diagnosis: Adenocarcinoma of the sigmoid.

This patient remained in the hospital. On March 15, 1941, rectal examination revealed metastases to the rectum. The patient was discharged on June 28, 1941. She is still alive at home, complaining of pain in the region of the sigmoid and rectum.

#### CONCLUSION

After a review of over 4,000 of these slides, two dominant blood patterns were noted: the first characterizes that of healthy persons and patients with

a variety of complaints; the second characterizes that of patients with cancer, and also those who have primary pernicious anemia, some with rheumatoid arthritis, and a few in late pregnancy. When treatment was instituted, however, in all but the cancer patients, the blood pattern changed to normal. In the cancer patients the typical "dotted-curtain" pattern persisted, except when total extirpation was achieved. Slides made on cancer patients up to within half an hour before death showed the same honeycombed appearance of the blood pattern. In patients in whom surgery had apparently removed all the tumor, and in some patients treated by radium or deep x-ray therapy, the blood pattern was found to return to normal after an interval of six weeks or more. No blood slides on patients who have remained well after operation for cancer have been made sooner than six weeks after operation. In one case of "cured" cancer of the stomach, the patient has had slides made weekly for a period of twenty-three months, and these have all been normal.

The erythrocyte count, the leucocyte count, and the hemoglobin appear to have no relationship with the carcinoma blood pattern on the slide. In my experience in cancer patients the blood picture changes relatively late. There is often a high red blood cell count and hemoglobin, with no indication of a malignant process. The changes in the blood picture occur only after clinical symptoms and signs have made it apparent that the disease is well advanced. Therefore, I have found this blood pattern test on the slide, when employed routinely, is of great value in suggesting, at least, the presence of carcinoma before it is discovered clinically or by roentgen ray.

Constant familiarity with a long series of slides enables the examiner to obtain a high percentage of correct readings. Erroneous interpretations have resulted, but the margin of error is small. This has happened when the technique has been faulty and the drops of blood have been too thick or have been smeared or not allowed to dry sufficiently; also the blood picture has changed somewhat when patients have had deep x-ray or radium therapy or repeated transfusions. We have already referred to those patients with pernicious anemia, etc. (Table I) in whom the reaction has been positive for a limited period.

The test is simple and can be done in the office by the examiner or his assistant with inexpensive equipment and with no loss of time during the course of a routine office examination. It is not offered as a *sine qua non*, but as an adjunct to be evaluated along with clinical and laboratory findings. In spite of a negative x-ray film, the test, if positive, should be carefully weighed and the patient closely watched. It may be an even more delicate index of the patient's condition than the clinical picture, leucocyte count, temperature curve, blood analysis, or x-ray film. The test is done routinely on every patient along with the general physical examination. Sometimes this cancer pattern has been the sole evidence of the presence of malignancy, and has led to a more thorough study of the patient.

Our results justify the continued use of this test as a detector of early malignant tumor formation.

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## BLOOD PRESSURE FLUCTUATIONS IN RESPIRATORY OBSTRUCTION\*

### EXPERIMENTAL OBSERVATIONS

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WITH THE TECHNICAL ASSISTANCE OF ROBERT S. BERKSON, DONALD W. HALL,  
MURL E. KINAL, AND ABIE S. LENZNER

IT HAS long been known that in normal persons and in animals there is a slight fluctuation of the systemic systolic blood pressure during quiet respiration.<sup>1</sup> In asthma patients, when the dyspnea is pronounced, it has been noted that the systolic blood pressure may fluctuate markedly during the respiratory cycle.<sup>2-5</sup> This fluctuation decreases as the asthmatic paroxysm subsides,<sup>3, 4</sup> and is usually within the normal limits of quiet respiration when the patient is asthma free.<sup>4</sup>

Recent literature reveals some experimental work with animals on the effects of various types of respiratory obstruction.<sup>6-9</sup> The reports include data on lung volume, blood changes, and the production of emphysema, but very few observations have been made of the effects of such obstruction on blood pressure.

The following experiments were carried out in order to determine whether blood pressure changes, similar to those observed in asthmatic patients, could be induced at will by obstructing respiration in animals and in man, and to detect a quantitative relationship, if any, between the amplitude of these changes and the degree of respiratory obstruction.

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## ANIMAL EXPERIMENTS

Cats were used anesthetized by chloralose rectally. Experiments were carried out on a number of animals, all of which responded similarly to the various procedures.

Simultaneous kymograph tracings were made of the carotid blood pressure, the intratracheal air pressure, the intrapleural pressure, and the respiratory movements. The blood pressure manometer was of mercury; the air pressure manometers were of water (see Fig. 1 for details of preparation of the cat). One to 3 c.c. of air were injected into the pleural cavity to prevent closure of the intrathoracic end of the blunted needle.

## 3mm. TRACHEAL Opening

## 4mm. TRACHEAL Opening

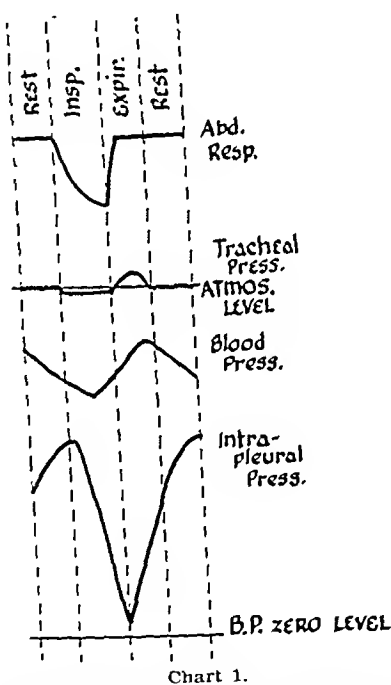


Chart 1.

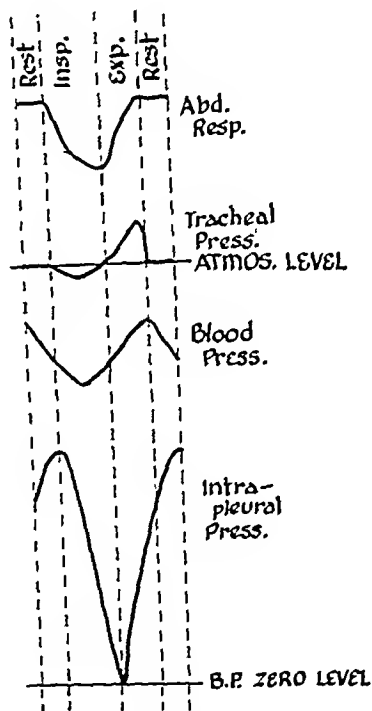


Chart 2.

Charts 1-5.—Rest, respiratory rest interval; Insp., inspiration; Exp., expiration. Scale drawings from Figures, bringing all simultaneous moments of time into the same vertical draw through the curves.

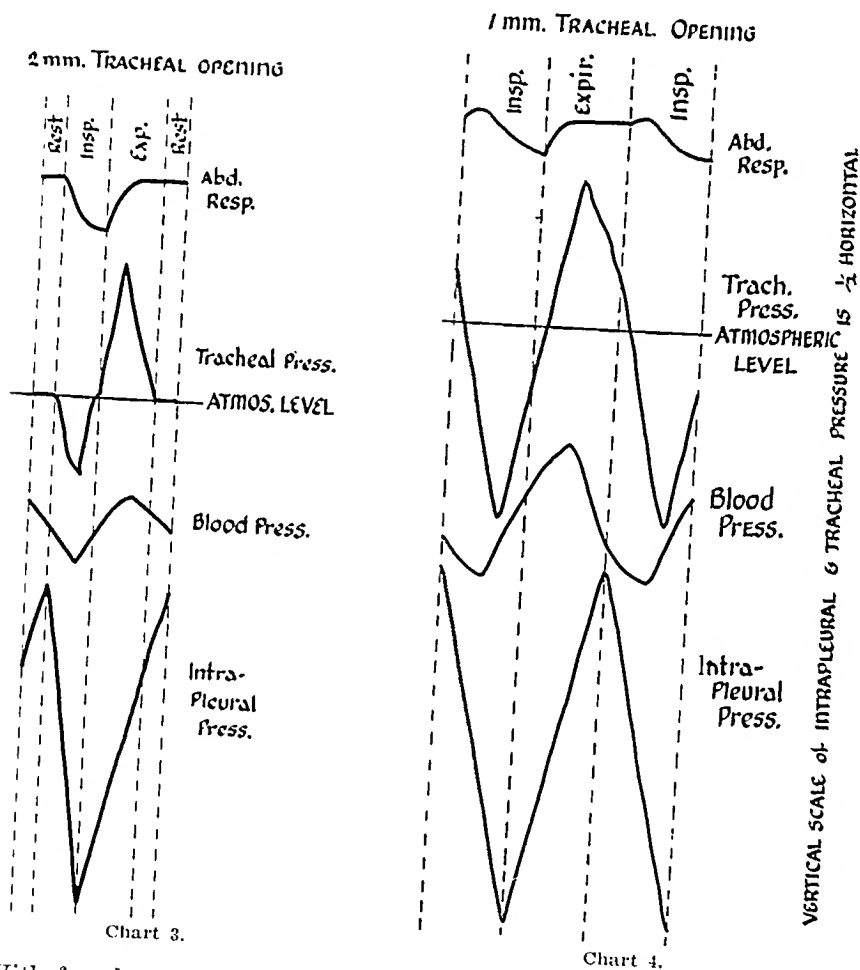
The open end of the T-shaped tracheal cannula was provided with a short rubber sleeve into which could be inserted short lengths of glass tubing with various-sized lumina. At the beginning of each experiment a glass tube with the lumen approximately equal to the diameter of the trachea was used, assuring free breathing. Successively smaller tubes were then inserted, introducing increasing obstruction to the respired air. Tracings of sufficient length were made with each size to secure reliable records. None of the cats survived with the 0.5 mm. tube in place. Tracings were continued until death.

The charts are scale drawings from the tracings, corrected so as to bring all points in any vertical line drawn through the tracings into the same moment

of time. The downstroke in the three respiratory tracings represents inspiration; the upstroke, expiration. The time line, with five-second intervals, lies at zero blood pressure level.

The following results are from continuous observations on a single cat, a healthy female, weighing 3.2 kg., anesthetized with 0.32 Gm. of chloralose.

A study of the tracings, and of the charts developed from them, will show several significant things.



With free breathing (4 mm. opening) there was a respiratory fluctuation of the blood pressure, amounting to about 4 mm. Hg. When the 3 mm. and 2 mm. openings were inserted, this fluctuation was increased slightly to about 5 mm. Hg. As would be expected, the fluctuations of the intratracheal and intrapleural pressures during respiration also increased somewhat, the increase being more noticeable in the intratracheal pressure, and with the 2 mm. opening. With the latter opening in place there was moderate respiratory obstruction.

The low point of the blood pressure, with free breathing (4 mm. opening) and with the 3 mm. and 2 mm. openings in place, occurred during inspiration, and the high point occurred during the latter part of expiration.

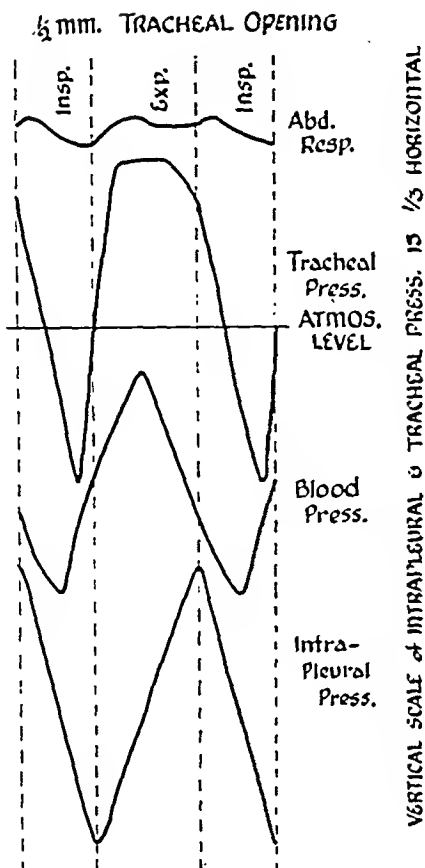


Chart 5.

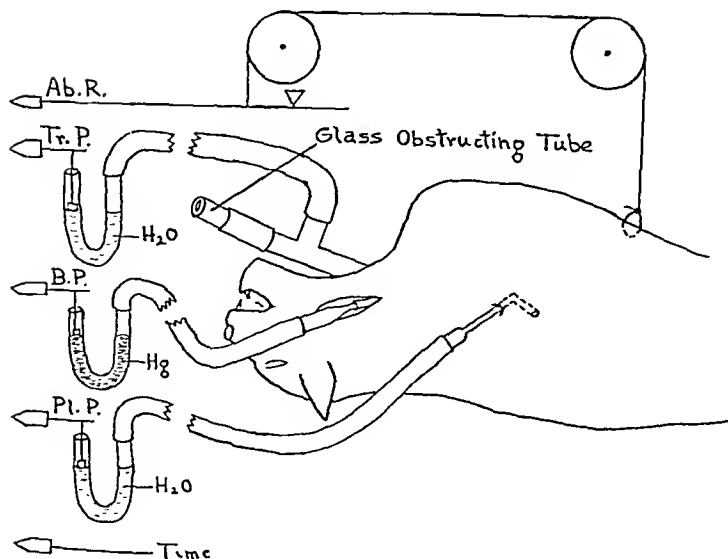


Fig. 1.—Preparation of the cat.

FIGS. 1-5.—Ab. R., abdominal respiration, mechanical; Tr. P., intratracheal pressure; B.P., blood pressure, carotid; Pl. P., intrapleural pressure; Time signal indicates five-second intervals and is at blood pressure zero.

When the 1 mm. opening was inserted, inducing marked respiratory obstruction, there was an immediate increase of the blood pressure fluctuation to 10 mm. Hg. This was raised still further to 15 to 20 mm. Hg when the 0.5 mm. opening was put in place. This smallest opening presented very marked obstruction to the respired air, causing the death of the cat from asphyxia eight or ten minutes later. The low point of the blood pressure still occurred during inspiration, and the high point occurred during expiration with both of these openings, although the exact location of these points cannot be precisely determined due to the wide swings and to the inertia of the water manometers used to record the intratracheal and intrapleural pressures.

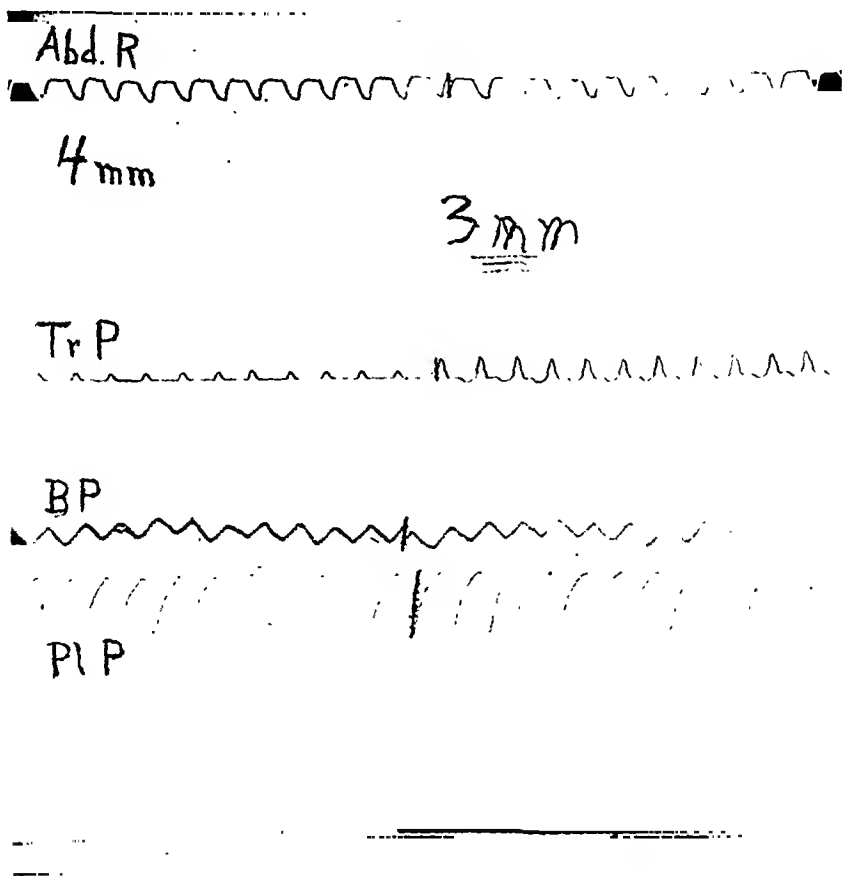


Fig. 2.

With the two smallest openings in place there was a tremendous swing of the intratracheal air pressure during inspiration and expiration, and a correspondingly large swing of the intrapleural pressure. As indicated by these two respiratory tracings, the intrathoracic pressure is greatly reduced during inspiration, and greatly increased during expiration.

#### SUMMARY OF FINDINGS ON CATS

The normal respiratory fluctuation of blood pressure in the anesthetized cat was increased very little when slight or moderate obstruction was presented

to the respired air at the trachea. When greater degrees of obstruction were created, the amplitude of this fluctuation increased two to four times; i.e., beyond a certain point, the greater the obstruction, the greater the blood pressure fluctuation.

The maximum and minimum blood pressure fluctuations occurred in the same phases of the respiratory cycle, whatever the degree of respiratory obstruction; i.e., the low point occurred during inspiration, and the high point during expiration.

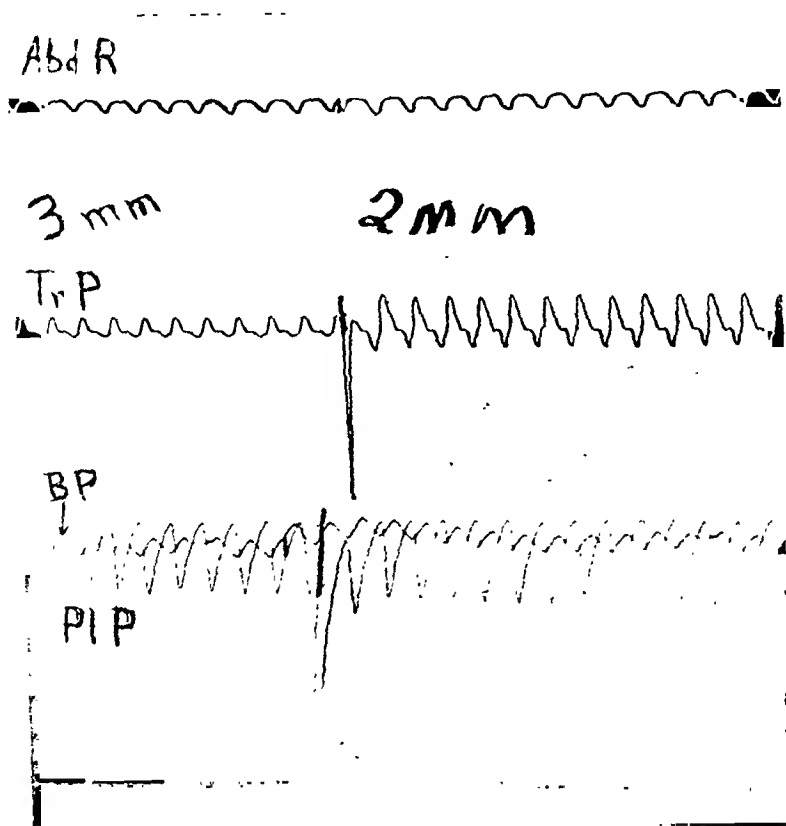


Fig. 3.

#### HUMAN EXPERIMENTS

In human beings fluctuations of the systolic blood pressure, synchronous with respiration, may be observed with considerable accuracy, using an ordinary sphygmomanometer. The 13 cm. cuff about the upper arm is inflated to above the systolic pressure. It is then slowly deflated (not over 2 or 3 mm. Hg per breath) while the respiratory movements are watched. At the high point of the systolic respiratory fluctuation a few arterial beats will be heard in the antecubital space by auscultation, followed by a trough of silence. If the cuff pressure is further lowered slowly, beats will come through more and more until they are heard throughout inspiration and expiration. The pressure reading where they are just heard throughout both phases of respiration marks

the low point of the fluctuation. On quiet breathing in normal persons, the fluctuation of the systolic blood pressure amounts to between 2 mm. and 6 mm. Hg, with the high point during expiration, and the low point during inspiration.

TABLE I  
RESULTS OF BREATHING THROUGH CONSTRICTED MOUTHPIECE

| APERTURE                                    | TIME  | SYSTOLIC B.P. |             | FLUCTUATION | DIASTOLIC |
|---|-------|---------------|-------------|-------------|-----------|
|   |       | EXPIRATION    | INSPIRATION |             |           |
| Subject William F.                          |       |               |             |             |           |
| Full  | 11:35 | 122           | 120         | 2           | 74        |
| $\frac{1}{4}$ " $\times$ $\frac{3}{4}$ "    | 11:37 | 114           | 112         | 2           | 80        |
| $\frac{3}{8}$ " $\times$ $\frac{5}{8}$ "    | 11:39 | 116           | 112         | 4           | 78        |
| $\frac{1}{2}$ " $\times$ $\frac{1}{2}$ "    | 11:42 | 114           | 114         | 0           | 78        |
| $\frac{1}{8}$ " $\times$ $\frac{1}{16}$ "   | 11:44 | 130           | 102         | 28          |           |
|   |       | 138           | 98          | 40          |           |
|   |       | 150           | 120         | 30          |           |
|   |       | 150           | 120         | 30          |           |
| No cyanosis noticeable                      |       |               |             |             |           |
| Subject Walter F.                           |       |               |             |             |           |
| No mouthpiece                               | 12:14 | 134           | 130         | 4           | 76        |
| $\frac{3}{4}$ " $\times$ $\frac{3}{4}$ "    | 12:16 | 120           | 116         | 4           | 80        |
| $\frac{1}{8}$ " $\times$ $\frac{3}{16}$ "   | 12:18 | 134           | 120         | 14          |           |
|   |       | 140           | 122         | 18          |           |
| $\frac{1}{8}$ " $\times$ $\frac{1}{16}$ "   | 12:20 | 150           | 114         | 36          |           |
| Very slight cyanosis with smallest aperture |       |               |             |             |           |
| Subject A. F. L.                            |       |               |             |             |           |
| No mouthpiece                               |       | 115           | 112         | 3           | 76        |
| $\frac{1}{16}$ " $\times$ $\frac{1}{4}$ "   |       | 135           | 122         | 13          | 78        |
| No cyanosis                                 |       |               |             |             |           |

TABLE II  
RESULTS OF HYPERVENTILATION WITH FREE BREATHING

|                            | SYSTOLIC B.P.          |             | FLUCTUATION | DIASTOLIC        |
|----------------------------|------------------------|-------------|-------------|------------------|
|                            | EXPIRATION             | INSPIRATION |             |                  |
| Subject A. F. L.           |                        |             |             |                  |
| Quiet respiration          | 115                    | 112         | 3           | 76               |
|                            | 124                    | 122         | 2           | 80               |
| Hyperventilation           | 124                    | 122         | 2           |                  |
|                            | 120                    | 117         | 3           |                  |
| Subject A. R. R.           |                        |             |             |                  |
| Quiet respiration          | 123                    | 120         | 3           | 74               |
| Hyperventilation           | 108                    | 104         | 4           |                  |
|                            | 104                    | 98          | 6           |                  |
| Subject W. F.              |                        |             |             |                  |
| Quiet respiration          | 130                    | 126         | 4           | 86               |
| Hyperventilation           | 140                    | 134         | 6           |                  |
|                            | 134                    | 130         | 4           |                  |
|                            | Rest (quiet breathing) |             |             |                  |
| Hyperventilation           | 126                    | 120         | 6           |                  |
| Hyperventilation continued | 118                    | 104         | 14          |                  |
| for one to two minutes     | 118                    | 106         | 12          | Slight dizziness |

Healthy young medical students in their twenties were used for the following experiments, using the foregoing method for determining the fluctuation of the systolic blood pressure. The subjects were recumbent.

With the nose closed with a clip and a wide rubber mouthpiece, bearing a thumb-screw pinchcock, held tightly in the mouth, the respiratory fluctuations of the systolic blood pressure were determined, first on free breathing, then with the mouthpiece narrowed in successive stages.

With the smaller apertures there was marked obstruction to respiration, and the respiratory rate was slowed to permit the passage of the tidal air. In all three subjects the low point of the blood pressure fluctuation came during inspiration, and the high point during expiration, both in free breathing and with all degrees of obstruction.

Since the respiratory efforts in breathing through small apertures resembled those of hyperventilation, blood pressure readings were made on healthy subjects with unobstructed breathing, both during quiet respiration and during voluntary hyperventilation.

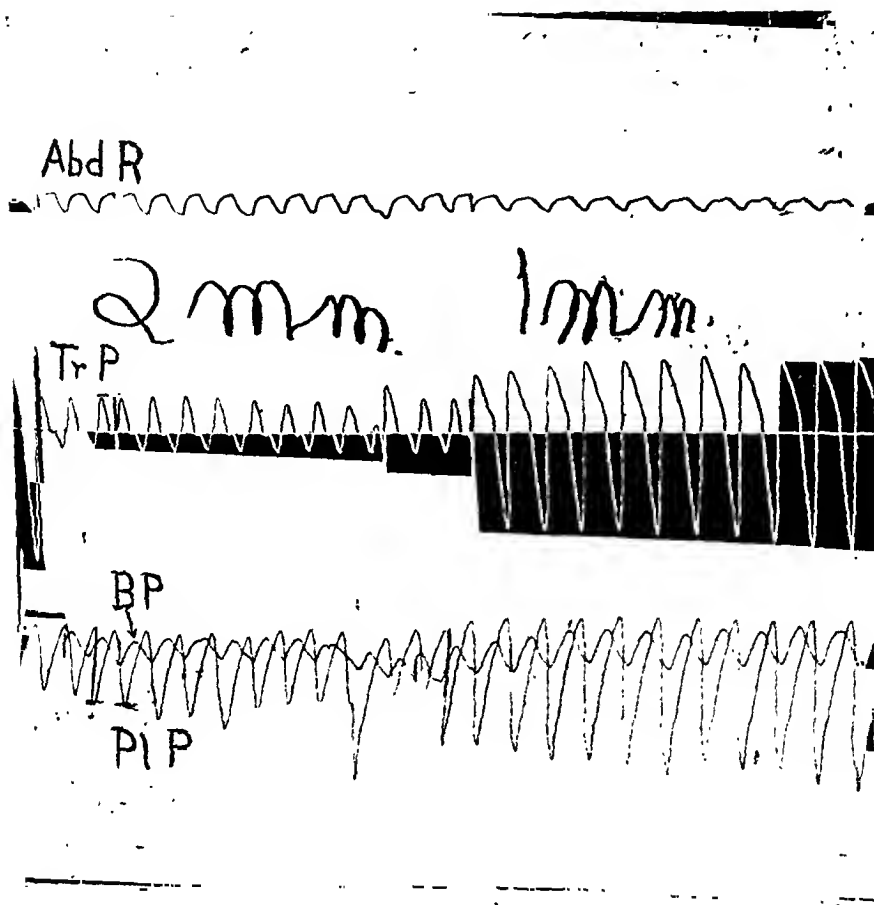


Fig. 4

As with the experiments with obstruction, likewise in hyperventilation, the low point of the systolic blood pressure fluctuation occurred during inspiration, and the high point during expiration.

Obstruction to respiration at the mouth caused an increase in the respiratory fluctuation of the systolic blood pressure roughly parallel to the degree of obstruction, as in the cat experiments. Hyperventilation alone, on the other hand, caused no increase in this blood pressure fluctuation in two subjects. In the third there was a moderate increase after hyperventilation had continued from one to two minutes.

## DISCUSSION

Prinzmetal<sup>8</sup> showed that the normal, slightly negative intrapleural pressure became more negative in asthmatic patients during the paroxysm, although he did not describe the fluctuations of this pressure during each respiratory cycle. In our cat experiments the intrapleural pressure fluctuated to an increasingly greater negativity during inspiration and showed an increasing, though less marked, movement toward the positive side during expiration, as the tracheal obstruction was increased.

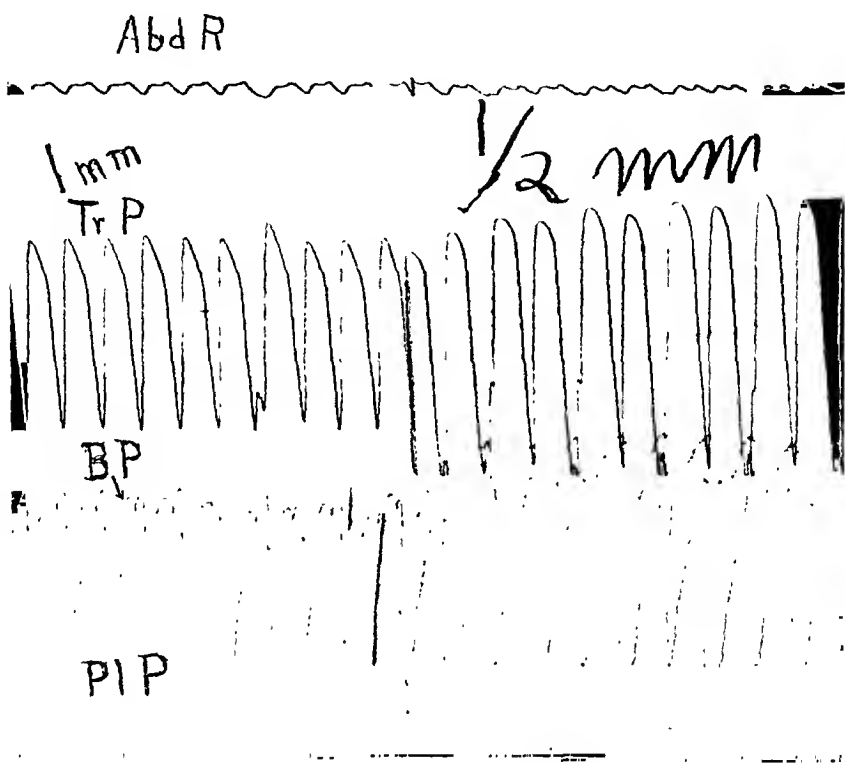


FIG. 5.

It would seem, therefore, that the fluctuations of the systolic blood pressure under respiratory obstruction, observed in our experiments, are closely related to the concurrent changes of the intrathoracic pressure during the respiratory cycle, and that their amplitude reflects roughly the degree of respiratory obstruction.

It would be unsafe to apply directly the results of these animal and human experiments to an explanation of the respiratory fluctuations of the systolic blood pressure observed in asthmatic patients, since the site of the obstruction to respiration differs in the two cases. Nevertheless, there is obstruction to breathing in bronchial asthma; and it has been shown<sup>8</sup> that the intrathoracic pressure deviates from the normal in this condition. Moreover, unpublished



observations which I have made show that the blood pressure fluctuations in asthma closely resemble those seen in our present experiments, both in their timing during the respiratory cycle, and in the parallelism between their amplitude and the degree of respiratory embarrassment. The assumption to be drawn, therefore, is that forces are in action in bronchial asthma, similar to those in the experiments here described and that these forces have a causal relationship to the observed blood pressure fluctuations.

### CONCLUSIONS

#### *Cat Experiments*

1. The greater the obstruction at the trachea to passage of the respired air, the greater the fluctuation of the blood pressure during the respiratory cycle.
2. Whatever the degree of respiratory obstruction, the high point of the blood pressure occurs during expiration, and the low point during inspiration.

#### *Human Experiments*

1. Marked respiratory obstruction at the mouth induces greatly increased respiratory fluctuation of the systolic blood pressure.
2. With this obstruction in place the high point of systolic blood pressure occurs during expiration, and the low point during inspiration.

I wish to express my appreciation to Dr. Frederick Reece Griffith, Jr., Professor of Physiology, University of Buffalo School of Medicine, for much encouragement and advice, and for his hospitality in making available for this work the facilities and resources of the laboratories of his Department.

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# THE TREATMENT OF SEVERE PERIODIC HEADACHES WITH "DESENSITIZING" DOSES OF PROSTIGMINE\*

A NEW CONCEPTION AND TREATMENT OF CERTAIN TYPES OF HEADACHES  
INCLUDING THOSE DESIGNATED AS MIGRAINE AND HISTAMINE HEADACHES

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OF THE many ills to which humanity is heir, headache is one of the most common. Even after arbitrarily dismissing those types due to increased intracranial pressure (brain lesions and hypertension), sinusitis, anemia, and the so-called "nervous" headaches, there still exists an extremely large group of periodic headaches loosely termed "migraine."

Brickner<sup>1</sup> has described "migraine headache" as a severe periodically recurring, unilateral headache which lasts from a few hours to several days, and is often accompanied by nausea and vomiting. Not infrequently the attack is preceded by an aura, consisting of some form of visual disturbance, and in many instances a familial history of migraine or allergy is present. In women the attacks frequently occur at the menstrual periods.

A considerable number of these "migraine" headaches have recently been reclassified by Horton and others<sup>2</sup> as histamine cephalalgias because they can be reproduced by histamine injections and can be controlled by desensitizing the patient with small but increasing doses of histamine. Typically, this new class of headache is characterized by a very severe unilateral pain of a burning, boring nature, which may involve the same side of the face. The conjunctiva on this side is often injected and the face is usually flushed. In general, the attacks occur more frequently, but are of shorter duration than the migraine type—lasting from fifteen minutes to several hours. Further points of difference exist in that they are not preceded by auras, vomiting is rare, and they are usually confined to the older age group.

The present work has been motivated by the desire (1) to extend the group of vascular headaches described by Horton to all those manifesting a marked skin reaction to histamine or acetylcholine; (2) to demonstrate a new method of producing these headaches; and (3) to present a new, very simple and extremely encouraging method of treating them.

The original stimulus to the present study was supplied by a chance observation in a patient with a typical histamine headache, as described by Horton. Several weeks subsequent to the cessation of histamine desensitization therapy,

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this patient suffered an attack which was aborted with remarkable promptness by the administration of one tablet of prostigmine bromide. Some time later, when his desensitization had apparently lapsed and he was on the verge of another headache, similar treatment with prostigmine produced a typical, very severe, cephalalgia, accompanied by fullness in the back of the head, diarrhea, abdominal cramps, and a mild attack of wheezing. It would appear, therefore, that prostigmine was able to duplicate in this patient with a typical "histamine cephalalgia," the actions of histamine in both the desensitized and sensitive phases. This observation was deemed of particular significance in view of the fact that Horton and others,<sup>2</sup> in summarizing his excellent work, stated, "The spectacular manner in which patients respond indicates that histamine treatment is as specific for this syndrome as insulin is in the treatment of diabetes mellitus."

Another hint that histamine might not be the only factor involved in this type of headache was demonstrated by another patient who failed to respond—even after two hours—to epinephrine (0.5 c.c. of 1:1,000 solution), whereas 1 c.c. of bellafolin (atropine-like in action) promptly alleviated the attack. To further confuse the issue, a preparation of histaminase (torantil), which effectively served to decrease the incidence of attacks in this patient one year ago, proved to be valueless in recent months.

In the light of these observations it was decided to investigate the effect of prostigmine on headaches of the migraine and histamine types with a view toward expanding the present knowledge of the causal relationships and the etiologic factors underlying these headaches. Accordingly, a series of 60 cases of headaches were carefully analyzed, and for the purpose of convenience were divided, symptomatically, into four principal groups. Some variations existed within each group since, obviously, though the same neurobiochemical mechanism might conceivably be operative in every case, the symptomatology was dependent upon such variables as the psychic, somatic, and endocrinologic factors. The groups were divided as follows:

Group I consisted of 20 patients with more or less typical histamine cephalalgias, who frequently manifested, in addition, such symptoms as fullness in the head, ears, and back of the neck, pain in the muscles of the neck, and dizziness.

Group II consisted of 25 patients with the "migraine" type. The headaches in this group were of longer duration than those in group I and were frequently preceded by auras and accompanied by vomiting. The patients were of the younger age group with headaches since childhood and frequently with familial histories of allergy, migraine, or premature graying of the hair.

Group III consisted of 8 patients who complained of a constant tightness and fullness in the back of the neck which did not alternate with the headaches described by Horton. Dizziness was a prominent feature in these cases, and occasionally there were full-blown attacks of Ménière's syndrome. In 2 of these patients the headaches were previously ascribed to hypertension, although the systolic pressure was 150 to 160 mm.

Group IV consisted of 7 patients with hypertension of whom 3 had systolic blood pressure of 200 mm. or more, with pounding of the vertex of the skull and fullness or tightness in the neck muscles. The remaining 4 were in the

hypotensive phase without cardiac decompensation. In these, the fullness in the head and ears, and the tightness of the neck muscles were very marked.

The common denominator in all the above groups was a marked cutaneous sensitivity to histamine and/or acetylcholine. All patients reacted markedly to 0.01 mg. of histamine (0.01 c.c. of a 1:1,000 solution) injected intradermally, by exhibiting a large wheal, pseudopodia, and an erythematous flare of 2 or more inches.\* Many also gave a marked reaction to 1 mg. of acetylcholine (0.02 c.c. of a 1:20 solution). Indeed, in some instances, sensitivity to acetylcholine was more pronounced than to histamine. Many patients were also allergic to some antigen.

#### TREATMENT

The notable sensitivity to acetylcholine was regarded as presumptive evidence of the possibility that acetylcholine might be a factor in the production of these headaches. It was hoped, therefore, that these patients could be "desensitized"—through a mechanism parallel to that operative in histamine "desensitization"—by the oral administration of increasing doses of prostigmine bromide, a parasympathomimetic drug, which is known to mobilize the acetylcholine reserves of the body by inhibiting the action of the acetylcholine esterase. Accordingly, a 15 mg. tablet of prostigmine bromide was dissolved in one ounce of water, and the resulting solution was administered as follows: On the first day, 1, 2, and 3 drops were given at morning, noon, and night, respectively. The increase of 1 drop per dose was maintained until 30 drops was reached, following which 30 drops were given each day for one week and then three times per week for an indefinite period. Occasionally, when it was felt that the results could be improved, the dose was increased to 40 drops. A few of the patients were given these drops in a slower fashion, e.g., the same dosage was given morning and night, and was increased by one drop each day until the dose of 30 drops was reached. Then the usual procedure was followed. This method was used when it was felt—on the basis of skin tests—that the patient was unduly sensitive to histamine or acetylcholine. For the purpose of uniformity in this experiment the majority of the patients were given identical dropper bottles containing previously prepared solutions.

The patients were initially skin tested with histamine before the institution of treatment, and repeat tests were made at approximately weekly or fortnightly intervals. Some were also tested with acetylcholine. Care was taken to swab the skin lightly with alcohol before the intradermal injection because vigorous rubbing in itself could produce a reaction in sensitive persons. A 26 gauge Schick needle with a tuberculin syringe was used for the test. The test was read arbitrarily five minutes after injection, traced on cellophane with a skin pencil, and retained for future records.

#### RESULTS

Significantly, in 40 per cent of the cases the skin reaction to injected histamine decreased, i.e., the size of the wheal was reduced, and the flare dimin-

Since nearly all persons give some reaction to intracutaneously injected histamine, it has been necessary to employ this criterion for a marked reaction. (Atkinson, M.: Observations on Etiology and Treatment of Ménière's Syndrome, J.A.M.A. 116: 1753, 1941.)

ished or disappeared by the treatment with "desensitizing" doses of prostigmine. Often in the initial histamine skin test there was marked localized itching, a symptom which was not evident in subsequent skin tests after the patients developed tolerance. There was no correlation, however, between the size of the wheal at a given time and the measure of relief. Of especial significance was the fact that the acetylcholine wheal and flare were always reduced in size following treatment. After one week the time taken for disappearance of the acetylcholine flare from the skin was usually halved.

TABLE I

| GROUP | TYPE  | LITTLE OR NO RELIEF | MODERATE RELIEF | MARKED TO COMPLETE RELIEF | TOTAL NUMBER OF CASES |
|-------|---|---------------------|-----------------|---------------------------|-----------------------|
| I     | "Histamine" headache  | 2                   | 1               | 17                        | 20                    |
| II    | "Other migraine types" of headache  | 2                   | 1               | 22*                       | 25                    |
| III   | Fullness in back of neck and dizziness + occasional attacks of Ménière's syndrome | 0                   | 0               | 8                         | 8                     |
| IV    | Hypertension with fullness in back of neck. Experimental                          | 0                   | 3               | 4                         | 7                     |

\*Since the acceptance of this paper, 11 (50 per cent) of these patients have reverted to the "moderate relief" designation. The reason for this is not clear but is being investigated.

All the patients exhibiting definite periodic headaches, including those similar to the headache described by Horton, and those usually classed as migraine were relieved in large measure. The relief was definite, dramatic, and in many cases complete, for the duration of time so far observed. The results are recorded in Table I. The figures are based on patients' estimates of relief of their headaches, and are, therefore, rather arbitrary. Several persons independently observed that even though a mild headache did occur, it could be dispelled in a few minutes by taking 15 drops of the solution.

In the patients with hypertension, that part of the headache described as fullness in the head, with the complaint that the head was twice the normal size, and the feeling of tightness of the neck muscles were relieved in several cases. The part ascribable to the hypertension, i.e., the pounding of the vertex of the skull, was not improved. The blood pressure was not reduced by this medication, but some patients with hypertension had only the fullness of the head and felt well once this was gone. The patient with hypertension in the hypotensive phase was usually completely relieved of symptoms, although the blood pressure did not rise.

#### CASE REPORTS

CASE 1.—C. M., male, aged 31 years, had a history of three years of severe, unilateral, burning and boring type of headaches, which lasted from several hours to a full day. These occurred about twice a week. There was no vomiting or aura. The patient's eyes teared, and his face appeared flushed on the side of the headache. For two months prior to treatment the headache appeared about four or more times a week and completely disabled him. The patient noticed that alcohol, even in slight quantities, could bring out a severe headache.

During the past year he has had three attacks of fullness in the back of the neck, coupled with dizziness that lasted all day. His previous history revealed a healed tuberculosis. His father has osseous tuberculosis. On May 25, 1941, the patient was put on prostigmine in desensitizing doses. On June 1 he had a slight headache over the whole forehead, but no unilateral headache. On June 8 he had one mild headache over the whole forehead, but again he had no unilateral headache. On June 15 he had one mild unilateral headache. The patient was last heard from on Oct. 1, 1941, at which time he stated that he had had no more unilateral headaches. Occasionally, after taking a drink of liquor, he developed a sensation as if a headache were about to come, but it never materialized. He was told to continue the medication. The histamine wheals are shown in Fig. 1.

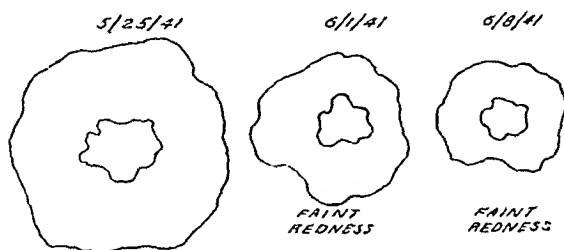


Fig. 1.—Histamine wheals (one-fourth actual size). Case 1, C. M.

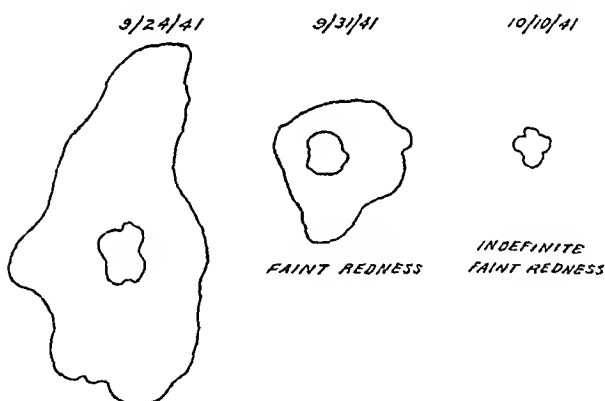


Fig. 2.—Acetylcholine wheals (one-fourth actual size). Case 2, L. P.

CASE 2.—L. P., female, aged 30 years, gave a history of severe unilateral headaches since childhood. The headaches usually lasted for about eight hours and culminated in vomiting. There was no lacrimation or flushing of the face. Occasionally there were some preheadache phenomena of slight blurring of vision. The only time in recent years that the patient has been free of headaches was during her two pregnancies, and a short interval when histaminase appeared to diminish the frequency of attacks. These severe headaches had been occurring at the rate of three to four times a week during the month previous to institution of treatment. They were especially frequent during shopping tours. The patient was also extremely reactive to soap, large blotches appearing on her skin after using any soap. The family history revealed that her brother has severe hay fever, and her sister has many attacks of vomiting and unilateral headaches that are not severe and last a short time. Her physical examination revealed a blood pressure of 120/90, normal eye grounds, normal refraction of the eyes, and normal ear, nose, and throat findings. On Sept. 24, 1941, following recovery from a very severe headache, she was skin tested with acetylcholine intradermally and prostigmine bromide was started in "desensitizing" doses. On September 31 she reported that

she had had no headache for a week, though at one time during this week she experienced some slight blurring of vision which did not, however, result in the usual severe headache. On October 5 she experienced a slight frontal headache, which did not last long and yielded readily to one aspirin tablet. When she was skin tested on October 10, she stated that she had had no severe unilateral headaches since the treatment. She also volunteered that she had ceased to be sensitive to soap. She was last seen on Dec. 1, 1941, and at that time she stated that she had not had the severe, unilateral type of headache that she had experienced before treatment. However, on two occasions she experienced an extremely mild unilateral fullness of the head but it did not incapacitate her. She was urged to continue the treatment. The acetylcholine wheals are shown in Fig. 2.

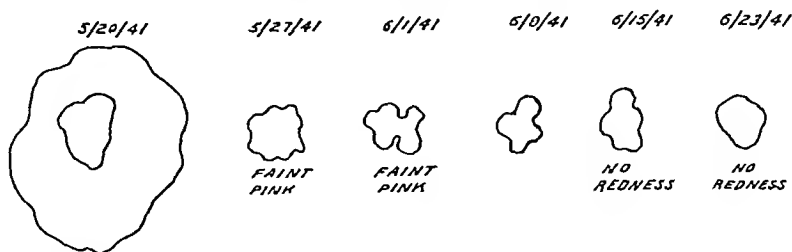


Fig. 3.—Histamine wheals (one-fourth actual size). Case 3, E. H.

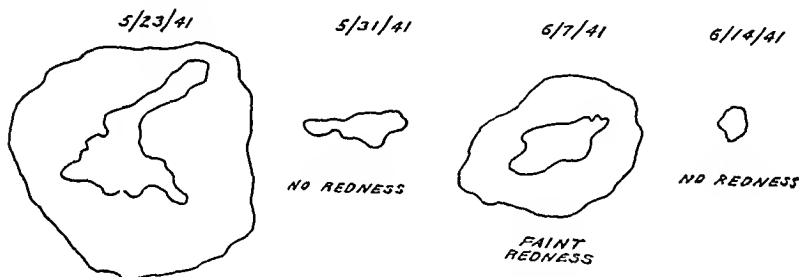


Fig. 4.—Histamine wheals (one-fourth actual size). Case 4, F. C.

CASE 3.—E. H., male, aged 27 years, stated that for three years he had frequent attacks of fullness of the head, dizziness, and tinnitus. In addition, practically every day he had the feeling of a "clouded" head, that reached a maximum in the afternoon. He was previously told that his blood pressure was 160, and that his headache was due to this. No treatment seemed to be of any avail. He presented an endocrine problem because of a voluminous supply of hair all over his body, coupled with underdeveloped genital organs. The physical examination revealed a blood pressure of 140/90, the eye grounds were normal, and the refraction was normal. On May 25, 1941, prostigmine was started in "desensitizing" doses. On June 1 the patient reported that he had had no fullness of the head at any time during the preceding week. On June 8 he stated that slight fullness was present on two days during the past week. He was told to increase the dosage to 40 drops and then take 40 drops a day. The blood pressure was 142/90. On June 15 the patient reported no fullness of the head or dizziness and stated that he felt very well. The blood pressure was 134/90. The patient was last seen on Sept. 15, 1941, at which time he did not have any fullness or clouding of the head. Several times during the past month he had a slight ringing in one of his ears; this vanished within a few moments. He has been told to continue taking the medication. The histamine wheals are shown in Fig. 2.

CASE 4.—F. C., female, aged 36 years, had attacks of fullness in the back of her neck, dizziness, and ringing in both ears for many years. These attacks occurred about two times a week for a few years. Her anamnesis had a marked hysterical taint; she was in constant

conflict with her husband because she desired to become pregnant and he refused to consider it. Previous history revealed that she was on a Bradford frame for many months in an orthopedic hospital, but the x-ray examination and physical signs revealed no osseous tuberculosis. On May 23, 1941, the physical examination revealed a blood pressure of 120/80, normal eye grounds, normal refraction, and normal nose and throat findings. On the same day prostigmine was started in "desensitizing" doses. On May 31 there was neither fullness nor dizziness. On June 7 the patient was well. By August 5 the prostigmine had been stopped one and one-half months without return of the symptoms in her head. On October 5, when she had not taken medication for approximately three months, there was slight return of the fullness of the head and dizziness. She was put on the "desensitizing" dosage of prostigmine. On October 12, 1941, when she was last seen, there was no return of the fullness since taking medication. The histamine wheals are shown in Fig. 4.

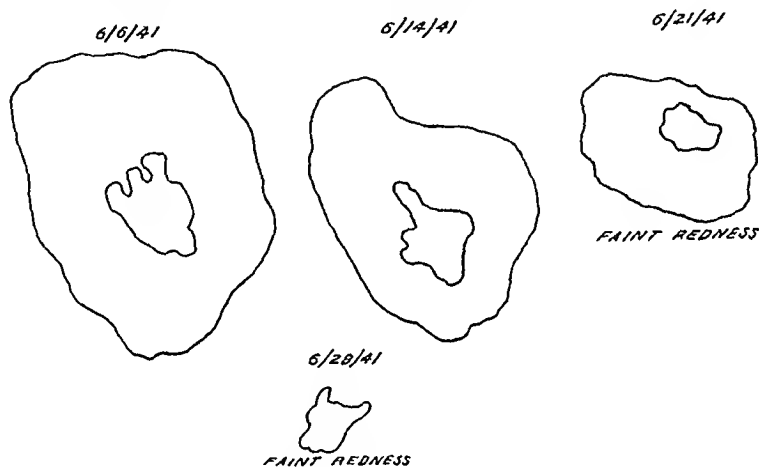


Fig. 5.—Histamine wheals (one-fourth actual size). Case 5, F. B.

CASE 5.—F. B., female, aged 41 years, came to the Jewish Hospital Clinic on June 6, 1941, with a history of constant fullness in her head, especially in the back of it, for the past four weeks. She also had occasional fullness in her ears. The head felt as if it were twice the normal size. The previous history revealed that on April 30, 1936, the blood pressure was 204/130, and that the cardiac outline was enlarged. On March 22, 1941, the blood pressure was 175/125, and on May 31, 1941, it was 150/100. Her menstrual periods were irregular. The physical examination revealed that she had a blood pressure of 145/100 at this visit and that there were normal eye grounds and normal refraction. On May 31 prostigmine was started in "desensitizing" doses. On June 14 she reported no fullness in the head. The blood pressure was 140/100. On June 21 she reported that there still was no fullness in the head. On June 28 she reported occasional frontal headaches but no fullness or "large head." On Oct. 1, 1941, when she was last seen, she reported that she felt perfectly well and had none of her original symptoms. Her blood pressure at that time was 150/100. The histamine wheals are shown in Fig. 5.

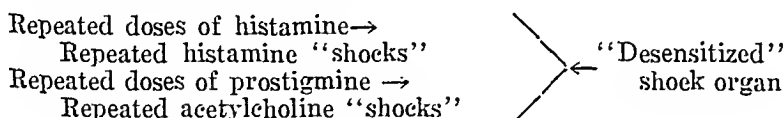
#### COMMENT

The results obtained in this study serve to extrapolate the well-known parallelism between histamine and acetylcholine<sup>3</sup> to the field of periodic headaches. Both of these substances are native to the body and both of them have been accused by different investigators of being the "allergic" substance. Histamine is produced by toxic or mechanical injuries of tissue cells, and acetyl-



choline results from the stimulation of the cell by the nerves. Acetylcholine would, therefore, appear to be the substance usually present unless some form of injury is presupposed. In addition, it would now appear that both histamine and acetylcholine may be causal factors in periodic headaches either at the same time or at different times. Certainly, both histamine and prostigmine are effective in the prophylactic treatment of these headaches.

These phenomena are of sufficient import to warrant the application of considerable thought to the question of the theoretical bases for the action of prostigmine. Admittedly, the data available are inadequate to permit the formulation, with any degree of certainty, of an all-embracing, coherent theory. Nevertheless, several explanations appear plausible enough to merit attention. The first of them is based on the known ability of prostigmine to liberate and to prolong the action of acetylcholine in the body by inhibiting the acetylcholine esterase. Thus the minute, controlled and ever-increasing quantities of acetylcholine liberated by the "desensitizing" doses of prostigmine may serve to administer mild repeated "shocks" to the hypersensitive organ responsible for the headaches, with the result that a relative tolerance or insensitivity is developed in this so-called "shock organ." This mechanism is sensibly analogous to that believed by many to be operative in histamine "desensitization." The analogy is perhaps best represented as follows:



Another explanation is based on the assumption that a person in the "sensitive" phase is in a state of autonomic or vasomotor imbalance with an ascendant parasympathetic system. This increased activity of the parasympathetic division accounts for the general hypersensitivity of the person toward both histamine and acetylcholine. Under such circumstances it may be postulated that prostigmine—though a para sympathetic stimulant in physiologic doses—may, in the minute desensitizing doses, increase the resistance of the parasympathetic division to both substances.

#### SUMMARY AND CONCLUSIONS

A series of 60 patients with periodic headaches with migraines and histamine cephalalgias predominating, were treated orally with gradually increasing doses of prostigmine. The extremely gratifying results which were obtained tend to prove (1) that in addition to histamine, acetylcholine is one of the factors involved in the production of these headaches; (2) that a similar neuro-biochemical mechanism is operative in many types of periodic headaches; (3) that prostigmine is as effective as histamine, and more convenient than it, in the treatment of these headaches.

We wish to express our appreciation to Hoffman-La Roche, Inc., for the materials used in this study.

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177 KINGSTON AVENUE

1523—11th AVENUE

## OBSERVATIONS ON THE EFFECT OF HISTAMINE PHOSPHATE ON CAPILLARY PERMEABILITY AND INFLAMMATION\*

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THE reactions of the skin to histamine are adequately described, and the observations of others are reviewed by Lewis.<sup>1</sup> When introduced into the skin in minute quantities, this poison "produces a characteristic series of independent phenomena comprising (a) local dilatation of the minute blood vessels by a direct action upon them; (b) a widespread dilatation of neighboring strong arterioles through the medium of a local reflex mechanism; and (c) increased permeability of the vessel walls by direct action."<sup>1</sup> Most investigators agree with these observations. In addition to these changes, Moon<sup>2</sup> has emphasized that there is stasis of blood with some diapedesis of red blood cells into the tissue spaces. The effects of histamine upon the systemic circulation are the same, and are due to the same mechanism as its local effects upon the capillaries of the skin.<sup>2</sup>

Bloom<sup>3</sup> in 1922 advanced the idea that the migration of leucocytes into the tissues in areas of inflammation may be due to histamine. Grant and Wood<sup>4</sup> were unable to demonstrate such an effect on the leucocytes of man, rabbit, and the frog. On the contrary, however, Wolf<sup>5</sup> and Findlay<sup>6</sup> showed that histamine acted as a positive chemotactic substance both in vitro and in vivo.

Best and McHenry<sup>7</sup> in 1931, in reviewing the role of histamine in inflammation, state that "the very interesting and fundamental question of the role of histamine or a histamine-like substance in the production of the phenomena observed in inflammation is still controversial. A thorough investigation of the subject is needed."

Interest in the role of histamine in the development of the local inflammatory reaction has been stimulated by the recent work of Menkin.<sup>8</sup> This investigator concludes from his experimental work that leucotoxine is the sub-

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stance responsible for the increase in capillary permeability and also for the localization of leucocytes in the local area. Menkin<sup>9</sup> says "in regard to the statement that various products of protein metabolism, such as amino acids, are capable of inducing leucocytic migration, it is interesting to note that observations with a number of representative amino acids (as well as histamine) have failed to reveal leucocytic migration." Furthermore, there is no evidence according to Menkin to support the contention that the permeability factor in inflammation is histamine.<sup>9</sup>

It has been shown that trypan blue following an intravenous injection localizes and concentrates in areas of inflammation in the skin of the rabbit only during a specific interval following the application of xylol to the skin. The greatest quantity of dye localizes and concentrates in the area of skin where the xylol is applied for the shortest time before the dye is injected. It has also been shown that the localization of leucocytes in areas of skin in the rabbit, showing macroscopic evidences of inflammation, is not determined by the time that trypan blue localizes and concentrates in the same tissue.<sup>10, 11</sup>

A problem is outlined to study the effect of histamine phosphate on capillary permeability and inflammation because there is some question as to the role that histamine may play in these processes. The technique is similar to that used in other studies on capillary permeability and inflammation.<sup>12-15</sup> Varying dilutions of histamine phosphate are injected intradermally preceding the intravenous injection of trypan blue. Observations are made on the localization and the concentration of dye in the histamine-injected areas of skin. Histologic studies are also made on the areas of skin injected with the histamine. Data are given on the changes produced in the number of circulating leucocytes following an intravenous injection of histamine.

#### METHODS AND MATERIAL

Adult rabbits are used. The hair is carefully shaven from the sides and the abdomen several days before the experiment is begun. The following dilutions of histamine phosphate\* are used: 1:1,000 (stock solution), 1:3,000, 1:5,000, 1:8,000, and 1:10,000. All dilutions are made in saline. Two-tenths cubic centimeter is injected intradermally. Several injections are made into different areas of the same animal. In some rabbits a single dilution is used while in others several dilutions are injected. Ten cubic centimeters of a 0.2 per cent solution of trypan blue in saline is injected intravenously following the last injection of the histamine. The time at which the histamine and the dye are injected is given in the individual experiments. Observations are made on the time of the localization and the concentration of the dye in the different areas of skin.

Areas of skin injected with the histamine are fixed in a 10 per cent solution of formalin immediately after the rabbits are killed. Paraffin sections are prepared and stained with hematoxylin and eosin.

Eight rabbits are used to study the effect of histamine on the number of circulating leucocytes. A standard technique is used for making these counts.

\*Supplied by Parke, Davis & Company.

The white blood cells are counted in each rabbit immediately before 0.4 c.c. of a 1:1,000 solution of histamine phosphate is injected intravenously into the marginal ear vein. The white blood cells are counted after one, two, four, five, seven, twenty-three, and twenty-eight hours. These data are analyzed statistically.

TABLE I

INTERVAL BETWEEN THE INTRADERMAL INJECTION OF HISTAMINE AND THE INTRAVENOUS INJECTION OF TRYPAN BLUE

| NUMBER OF RABBIT                      | MINUTES |    |    |    | HOURS |   |   |   |   |   |
|---------------------------------------|---------|----|----|----|-------|---|---|---|---|---|
|                                       | 0       | 15 | 30 | 60 | 2     | 3 | 4 | 5 | 6 |   |
| <i>1:10,000 Dilution of Histamine</i> |         |    |    |    |       |   |   |   |   |   |
| 967                                   | 0       | 0  | 0  | 0  | 0     | 0 | 0 | 0 | 0 | 0 |
| 968                                   | +       | +  | 0  | 0  | 0     | 0 | 0 | 0 | 0 | 0 |
| 969                                   | +       | 0  | 0  | 0  | 0     | 2 | - | - | - | - |
| 976                                   | +       | +  | +  | -  | -     | - | - | - | - | - |
| <i>1:8,000 Dilution of Histamine</i>  |         |    |    |    |       |   |   |   |   |   |
| 969                                   | +       | 0  | 0  | 0  | 0     | - | - | - | - | - |
| 976                                   | +       | +  | +  | -  | -     | - | - | - | - | - |
| 1011                                  | +       | ?  | ?  | 0  | 0     | 0 | 0 | 0 | 0 | 0 |
| 1016                                  | +       | 0  | 0  | 0  | 0     | 0 | 0 | 0 | 0 | 0 |
| 1017                                  | +       | 0  | 0  | 0  | 0     | 0 | 0 | 0 | 0 | 0 |
| <i>1:5,000 Dilution of Histamine</i>  |         |    |    |    |       |   |   |   |   |   |
| 969                                   | +       | 0  | 0  | 0  | 0     | - | - | - | - | - |
| 976                                   | 0       | 0  | +  | -  | -     | - | - | - | - | - |
| 1060                                  | +       | +  | +  | 0  | 0     | - | - | - | - | - |
| 1061                                  | +       | +  | +  | 0  | 0     | - | - | - | - | - |
| 1065                                  | +       | -  | +  | 0  | 0     | 0 | 0 | 0 | 0 | 0 |
| 1066                                  | +       | -  | +  | 0  | 0     | 0 | 0 | 0 | 0 | 0 |
| <i>1:3,000 Dilution of Histamine</i>  |         |    |    |    |       |   |   |   |   |   |
| 969                                   | +       | +  | 0  | 0  | 0     | - | - | - | - | - |
| 976                                   | +       | 0  | 0  | -  | -     | - | - | - | - | - |
| 1060                                  | +       | +  | +  | 0  | 0     | - | - | - | - | - |
| 1061                                  | +       | +  | 0  | 0  | 0     | - | - | - | - | - |
| 1065                                  | +       | -  | +  | 0  | +     | 0 | 0 | 0 | 0 | 0 |
| 1066                                  | +       | -  | +  | +  | 0     | 0 | 0 | 0 | 0 | 0 |
| <i>1:1,000 Dilution of Histamine</i>  |         |    |    |    |       |   |   |   |   |   |
| 784                                   | +       | +  | +  | +  | +     | + | + | + | + | + |
| 945                                   | +       | +  | +  | 0  | +     | + | + | + | + | 0 |
| 946                                   | +       | +  | +  | +  | +     | + | + | + | + | + |
| 969                                   | +       | +  | +  | +  | +     | - | - | - | - | - |
| 976                                   | +       | +  | +  | -  | -     | - | - | - | - | - |
| 804                                   | +       | +  | +  | +  | +     | + | + | + | + | + |
| 1060                                  | +       | +  | -  | +  | +     | - | - | - | - | - |
| 1061                                  | +       | +  | -  | +  | +     | - | - | - | - | - |

## EXPERIMENTAL

## OBSERVATIONS ON THE LOCALIZATION AND THE CONCENTRATION OF TRYPAN BLUE IN AREAS OF SKIN INJECTED WITH HISTAMINE

A group of rabbits are injected intradermally with a series of different dilutions of histamine at the following intervals before 10 c.c. of trypan blue are injected intravenously: six hours, five hours, four hours, three hours, two hours, one hour, thirty minutes, fifteen minutes, and immediately. The concentration of histamine injected and the location of the dye in the skin are shown in Table I. It is evident from these data that trypan blue localizes and

concentrates for less than thirty minutes in areas of skin previously injected with dilutions of histamine less than 1:3,000. When a 1:1,000 dilution of histamine is injected, the dye may localize and concentrate in areas of skin injected as long as five to six hours previous to the injection of the dye. Some of these rabbits are injected with only a single dilution of the histamine.

The skin at the site of the injection of the histamine usually becomes blanched immediately following an intradermal injection. This color persists for varying periods of time. The skin around this blanched area becomes hyperemic and apparently edematous. The macroscopic size of the skin lesion is influenced by the concentration of the histamine. The lesions may persist for six hours when a 1:1,000 dilution of histamine is injected. There is only an insignificant amount of blanching when a 1:10,000 dilution of histamine is injected. The hyperemia persists for only a short time.

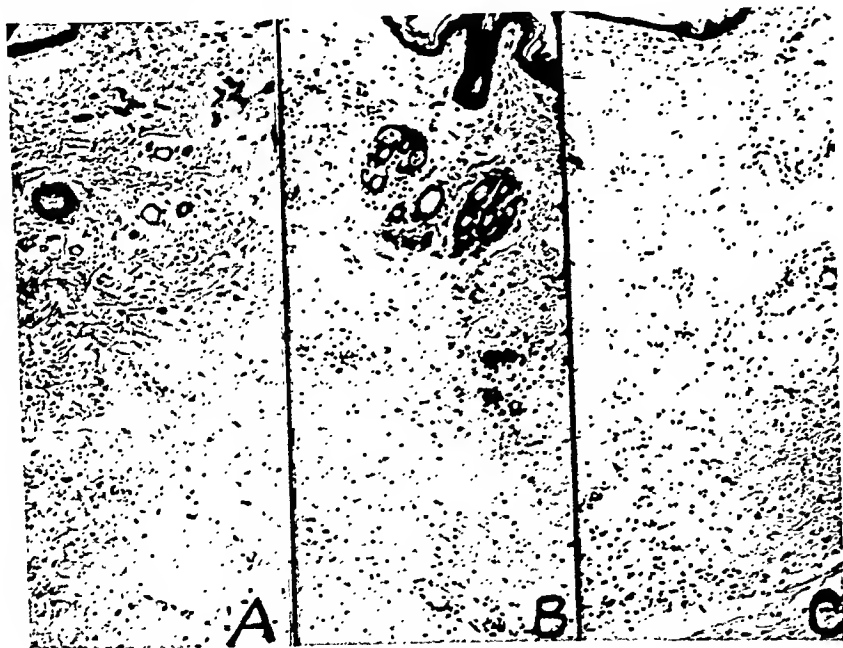


Fig. 1.—A, The subcutaneous tissue is slightly edematous. How much of the edema results from the fluid injected and how much from the effect of the histamine on the capillary walls cannot be determined histologically. This rabbit was injected intradermally with 0.2 c.c. of a 1:1,000 dilution of histamine phosphate for ten minutes before this section was removed.

B, In addition to some edema in the subcutaneous tissue there are few polymorphonuclear leucocytes. These are present especially around the small blood vessels. This is the same rabbit as shown in A. Two-tenths cubic centimeter of the 1:1,000 dilution of histamine phosphate was injected intradermally five hours before this section was removed.

C, Leucocytes and edema are present in the subcutaneous tissue. This is the same rabbit as shown in A and B. Two-tenths cubic centimeter of the 1:1,000 dilution of histamine phosphate was injected intradermally eight hours before this section was removed.

Trypan blue stains a ring of tissue blue around the blanched zone at the site of the intradermal injections. This dark blue zone may measure as much as 1 cm. The blue color then gradually fades as it extends peripherally. The entire area of skin becomes more or less uniformly blue when the most dilute solutions of histamine are injected. Trypan blue may fail to localize and to concentrate in areas of skin injected with a 1:3,000, 1:5,000, 1:8,000, and

1:10,000 dilution of histamine, although the skin is hyperemic. This occurs when the dye is given either thirty minutes or longer following the injection of the histamine.

#### HISTOLOGIC STUDIES UPON THE AREAS OF SKIN INJECTED WITH HISTAMINE

Sections of skin are removed from 5 rabbits injected with 0.2 c.c. of a 1:1,000 dilution of histamine at the following intervals before the animals are killed: 0 minutes, fifteen minutes, thirty minutes, one hour, two hours, three hours, four hours, five hours, and six hours. Sections of skin are also removed from 3 other rabbits injected with 0.2 c.c. of a 1:8,000 dilution of histamine at corresponding intervals before death.

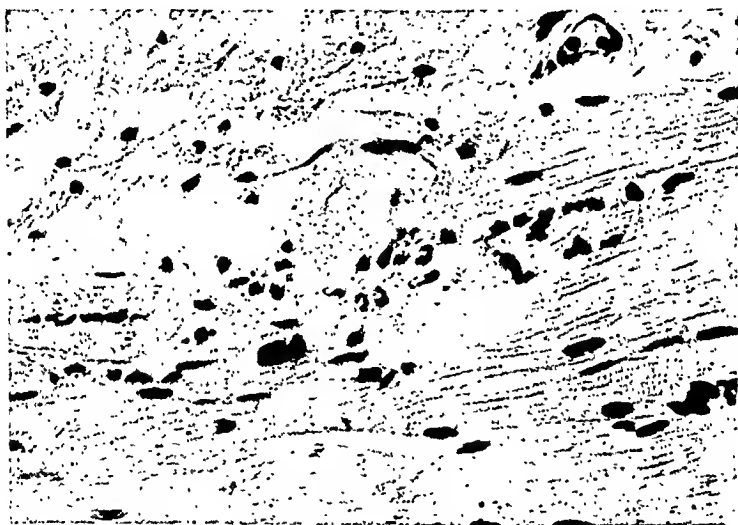


Fig. 2.—Small, focal areas of degeneration occur in the fibers of the cutaneous maximus muscle following the intradermal injection of 0.2 c.c. of a 1:1,000 dilution of histamine phosphate. Polymorphonuclear leucocytes infiltrate the area about the necrotic tissue. The histamine was injected five hours before this section was removed.

The histologic changes are essentially the same when a 1:8,000 and a 1:1,000 dilution of histamine is injected. There may be some difference in the extent of the lesion, but this factor is difficult to determine from these sections. It is also difficult to separate the effect of the volume of fluid injected from the edema fluid that results from the direct effect of the histamine on the capillaries. Polymorphonuclear leucocytes are present in the extravascular tissue usually within an hour following the injection of the histamine. The number of these cells apparently increases during the following four to five hours. After this time apparently the number of leucocytes decreases. Leucocytes usually infiltrate the corium and the subcutaneous tissues down to the cutaneous maximus muscle (Fig. 1). Sometimes there is a pink stain material in the subcutaneous tissue. It is considered to be coagulated serum.

There are small focal areas of degeneration in the cutaneous maximus muscle. It is impossible to determine accurately the time at which this lesion begins; however, the sections removed two hours after the histamine is injected

show unquestionable necrosis. This lesion is characterized by a swelling of the muscle fiber and a loss of the striations. The area appears glossy and stains deeply with eosin. In some of the sections the muscle fibers are broken and the margins are irregular. Polymorphonuclear leucocytes are present in large numbers around such muscle fibers (Fig. 2).

Four rabbits are injected subcutaneously with 0.2 c.c. of a 1:1,000 dilution of histamine at the following intervals before they are killed and the sections are removed: seven, six, five, four, three, two, and one hours. An attempt is made to inject the histamine into the cutaneous maximus muscle in these animals. This technique is unsatisfactory. The sections from this material show the same histologic changes as those observed in the other animals, except there is a more extensive lesion in the muscle of these animals than there is in the former group of rabbits.

#### EFFECT OF HISTAMINE PHOSPHATE ON THE NUMBER OF CIRCULATING LEUCOCYTES IN THE RABBIT

The data from these counts are given in Table II. The number of leucocytes in the circulating blood increases during the seven hours following the intravenous injection of histamine. The number of leucocytes in the circulating blood, however, returns to within the range of normal twenty-four hours following the injection of the histamine.

TABLE II

EFFECT OF HISTAMINE PHOSPHATE ON THE NUMBER OF CIRCULATING WHITE BLOOD CELLS IN THE RABBIT

| RABBIT NO.                 | 9:00<br>A.M. | 10:00<br>A.M. | 11:00<br>A.M. | 1:00<br>P.M. | 2:00<br>P.M. | 4:00<br>P.M. | 8:00<br>A.M. | 1:00<br>P.M. |
|----------------------------|--------------|---------------|---------------|--------------|--------------|--------------|--------------|--------------|
| 914                        | 9450         | 21600         | 17200         | 26550        | 11800        | 14350        | 16350        | 10450        |
| 915                        | 7750         | 13300         | 19650         | 14150        | 14450        | 15500        | 14500        | 12600        |
| 916                        | 10350        | 15450         | 16050         | 33300        | 19900        | 21350        | 22200        | 17950        |
| 917                        | 14200        | 15750         | 22600         | 30400        | 22100        | 13650        | 9950         | 14500        |
| 1012                       | 11450        | 12500         | 40800         | 41800        | 47400        | 13500        |              |              |
| 1013                       | 7800         | 29150         | 27200         | 12900        | 26250        | 12350        |              |              |
| 1014                       | 7600         | 17150         | 19800         | 13700        | 17850        | 17800        |              |              |
| 1015                       | 10150        | 6000          | 14600         | 25000        | 19100        | 14600        |              |              |
| Means                      | 9843         | 16362         | 22212         | 24725        | 22356        | 15387        | 15750        | 13875        |
| Differences in mean count* |              | +6519         | +12369        | +14882       | +12513       | +5544        |              |              |
| Value of $t_{\dagger}$     |              | 2.56          | 3.98          | 3.90         | 3.14         | 4.26         |              |              |
| Value of $t_{\ddagger}$    |              |               |               | 5.25         | 5.87         | 2.68         |              |              |

\*Differences in the mean leucocyte counts and the value of  $t$  for various hours.

$t$  must equal 2.145 or more for the increase to be statistically significant.

Histamine injected at 9:00 A.M. immediately following the blood count.

$\dagger$ : Value of  $t$  when the experimental animals are compared hour to hour—14 degrees of freedom.

$\ddagger$ : Value of  $t$  when experimental animals are compared with 32 control animals—38 degrees of freedom.

The values of  $t$  are calculated for these samples of 8 rabbits in order to compare the counts at 9:00 A.M., 10:00 A.M., 11:00 A.M., 1:00 P.M., 2:00 P.M., and 4:00 P.M. of the day of injection; and counts are made again at 8:00 A.M. and 1:00 P.M. on the following day.  $t$  must equal for 14 degrees of freedom 2.145 or more if the increase in the mean leucocyte count is significant. The

mean counts at the specified hours and the values of  $t$  are given in Table II. The mean leucocyte counts of the experimental animals are also compared with the mean leucocyte counts of 32 control animals, and the values of  $t$  for these comparisons are also shown in Table II. For 38 degrees of freedom  $t$  must equal 2.02 for the differences to be statistically significant.

#### DISCUSSION

It is evident from this study that histamine phosphate, when injected intradermally in the rabbit, acts as a chemotactic substance. This observation is confirmatory of the studies of Wolf<sup>5</sup> and Findlay;<sup>6</sup> however, it does not support the opinion expressed by Bloom<sup>3</sup> and Grant and Wood<sup>4</sup> as to the effect of histamine on the attraction of leucocytes.

The presence of necrosis in the cutaneous maximus muscle, following the intradermal injection of 0.2 c.c. of a 1:1,000 and 1:8,000 dilution of histamine, indicates that this substance may produce not only an increase in capillary permeability, but also a severe injury that culminates in necrosis. Because of this necrosis it becomes more difficult to ascertain the role of histamine in inflammation.

Trypan blue following an intravenous injection localizes and concentrates in areas of skin previously injected with histamine. The localization and the concentration of trypan blue in these areas are influenced by both the interval between the injection of the histamine and the injection of the dye, and the concentration of histamine. The skin, where 0.2 c.c. of a 1:1,000 dilution of histamine is injected, becomes blue within a period of five minutes when the dye is injected intravenously as long as six hours after the intradermal injection of the histamine. In contrast to this the skin does not show a greater concentration of dye than that in the untreated area, when an interval of thirty minutes elapses between the injection of a 1:10,000 dilution of histamine and the intravenous injection of the trypan blue. These observations suggest that histamine, when injected intradermally in the rabbit, produces a disturbance in the metabolism of the regional cells. Trypan blue following an intravenous injection localizes and concentrates in this area of skin only during the time that this abnormal metabolic process is present. The cells may return to the "so-called normal" very quickly, as indicated by the failure of trypan blue to localize and to concentrate in the skin when a 1:10,000 dilution of histamine is injected thirty minutes before the dye is given. The cells remain abnormal for at least six hours when a 1:1,000 dilution of histamine is injected. This interval during which trypan blue may localize and concentrate in areas of skin previously injected with histamine may be influenced by the total quantity of histamine given to the animal.

A specific period during which trypan blue localizes and concentrates in an area is the same phenomenon as previously observed with a group of irritants and a variety of circulating substances. Trypan blue, India ink, a virus, staphylococci, and staphylococcus antitoxin each localizes and concentrates in the skin only within a period of three hours following the local application of xylol.<sup>11</sup> When staphylococci are injected intradermally and trypan blue is given intravenously, the largest amount of the dye localizes in the area where



the bacteria are injected within a period of three hours previous to the injection of the dye.<sup>17</sup> In a discussion of these observations it has been suggested that "the process in inflammation referred to as increased capillary permeability represents a phenomenon well recognized in vital staining. The staining results from a metabolic disturbance in the epithelial cells, connective tissue cells, and endothelial cells of the small blood vessels."<sup>18</sup>

It is of interest to know that the polymorphonuclear leucocytes concentrate in areas of skin injected intradermally with histamine for a much longer period than that in which trypan blue localizes and concentrates in the same area. It has been pointed out in other studies that "the dye may localize and concentrate in tissue when there are no polymorphonuclear leucocytes and fail to localize and to concentrate when the subepithelial tissues are diffusely infiltrated with leucocytes."<sup>18</sup>

The data in this experiment show that there is a statistically significant increase in the leucocyte counts of rabbits injected intravenously with 0.4 c.c. of a 1:1,000 dilution of histamine phosphate. This increase occurs within one hour of the time of injection and continues for at least seven hours. These data are obtained by comparing the counts of the experimental animals with the counts from a group of 32 normal rabbits.

The demonstration of a leucocytosis following the injection of histamine phosphate corresponds to the observations of Moon.<sup>2</sup> He finds that histamine produces a leucocytosis in cats, monkeys, and human beings. Menkin,<sup>8</sup> however, considers that histamine is essentially inactive for producing an increase in the leucocytic level in the blood of both dogs and rabbits.

It is obvious that the phenomena that occur in the local areas of inflammation and in the circulating blood are complex. These experimental observations with histamine may be an index of some of these changes; however, one must be careful in interpreting them, since there appears to be a variety of unrelated substances, each of which may produce an increase in capillary permeability and a localization of leucocytes in the skin of the rabbit.

#### SUMMARY

Histamine phosphate, when injected intradermally in the rabbit, produced a change in the tissue cells that permits the localization and the concentration of trypan blue in the local area. This localization and concentration of trypan blue are influenced by both the concentration of histamine and the interval between the intradermal injection of the histamine and the intravenous injection of the dye.

Polymorphonuclear leucocytes localize in the subcutaneous tissues of the rabbit following an intradermal injection of histamine phosphate.

Necrosis occurs in the fibers of the cutaneous maximus muscle following the intradermal injection of histamine phosphate.

The number of white blood cells increases in the circulating blood following an intravenous injection of histamine phosphate. The number, however, returns to normal within a period of twenty-four hours.

I am greatly indebted to Dr. F. L. Roberts, Professor of Preventive Medicine, University of Tennessee, for his assistance in the statistical problems concerned in this study.

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# CLINICAL CHEMISTRY

## CHOLESTEROL STUDIES IN THE AGED\*

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NUMEROUS investigators<sup>1-11</sup> have studied the total cholesterol concentration and its partition, that is, the ratio of the free cholesterol, or the cholesterol esters, to the total cholesterol in the blood of persons below 60 years of age. Comparatively scant attention, however, has been paid to the cholesterol partition in the blood of normal persons past 60 years of age. A difference of opinion also exists as to whether advancing age affects the cholesterol values. In 1923 Parhon and Parhon<sup>12</sup> stated that the total cholesterol concentration in whole blood increased slightly with age. In 1924 Bing and Heckscher<sup>13</sup> found that the blood lipids were relatively low in youth and increased with advancing age. These observers stated that the upper and lower limits for the total cholesterol in the blood of normal aged persons were 260 and 125 mg. per 100 c.e., respectively. On the other hand, Page, Kirk, Lewis, Thompson, and Van Slyke<sup>4</sup> found that in a study of the lipids in the plasma of males of all ages there was no regular important change in any blood lipid fraction with advancing years. Similarly, Hunt<sup>14</sup> stated that the age of the patient does not affect the blood cholesterol values. It was, therefore, thought of interest to make a study of the blood cholesterol and its partition in the serum and whole blood of normal aged males and females.

### EXPERIMENTAL STUDY

The subjects chosen for this study comprised 76 males and 74 females. All were housed in the "Home" division of the institution, all were ambulatory, and all were on the normal institutional diet. Their ages ranged from 62 to 104 years and were distributed as outlined in Table I. The mean ages were found to be 77.1 years for males and 76.2 years for females.

Determinations were made of total and esterified cholesterol in both serum and in whole blood, using the method of Bloor and Knudson.<sup>7</sup> All determinations were made after a fasting period of at least eight hours in order to rule out any temporary lipemia due to ingestion of fatty materials. The free cholesterol was computed in each case by subtracting the esterified cholesterol from the total cholesterol, and this value, divided by the total cholesterol, gave the fraction existing in the free state.

Table II shows the distribution of the total cholesterol concentration in milligrams per 100 c.e. for both whole blood and serum for both sexes. Table III shows the distribution of the free cholesterol percentages for both whole blood and serum for both sexes.

\*From the Hospital and Home of the Daughters of Jacob, New York.  
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The values for the total cholesterol concentration in the blood of the male subjects in our study ranged from 180 to 340 mg. per 100 c.c. in serum, and from 158 to 316 mg. per 100 c.c. in whole blood. The range of cholesterol concentration in whole blood for females was from 149 to 329 mg. per 100 c.c., and in serum the lower and upper limits were 168 and 320 mg. per 100 c.c., respectively.

TABLE I  
AGE-FREQUENCY DISTRIBUTION OF SUBJECTS STUDIED

| AGE GROUP<br>(YEARS) | MALES | FEMALES | TOTAL |
|----------------------|-------|---------|-------|
| 60-65                | 4     | 0       | 4     |
| 65-70                | 8     | 14      | 22    |
| 70-75                | 19    | 19      | 38    |
| 75-80                | 21    | 25      | 46    |
| 80-85                | 12    | 10      | 22    |
| 85-90                | 7     | 5       | 12    |
| 90-95                | 5     | 0       | 5     |
| 95-100               | 0     | 0       | 0     |
| 100-105              | 0     | 1       | 1     |

The free cholesterol in males was between 16 and 64 per cent in serum and between 14 and 75 per cent in whole blood. The free sterol for females showed a variation between 17 and 67 per cent in serum and between 11 and 75 per cent for whole blood. The average total cholesterol values in whole blood were computed to be 219 mg. per 100 c.c. for males and 227 mg. per 100 c.c. for females, and in serum the average total cholesterol values were found to be 246 mg. per 100 c.c. for males and 232 mg. per 100 c.c. for females. The average value for free cholesterol in whole blood was 39 per cent for males and 50 per cent for females, while in serum the average free cholesterol was 37 per cent for males and 42 per cent for females. The over-all average for total cholesterol in whole blood was 223 mg. per 100 c.c.; with the free sterol amounting to 46 per cent. Averaging all the values for serum gave a total cholesterol of 238 mg. per 100 c.c. of which 39 per cent existed in the free state.

#### COMMENT

The total cholesterol and cholesterol partition were studied in a group of 150 so-called normal aged persons. According to Todd and Sanford,<sup>15</sup> the normal limits for total cholesterol estimated by the Bloor method were 165 to 200 mg. per 100 c.c. for plasma or serum with 30 per cent as the maximum normal free sterol. Bloor and Knudson<sup>7</sup> found that the total concentration of cholesterol was from 180 to 200 mg. per 100 c.c., "little difference being noted between the results for whole blood and serum if the normal ratio of esters to free sterol is maintained." They further stated that in serum the maximum normal percentage of free sterol encountered was about 40 per cent, and in whole blood the free cholesterol ran to a maximum of 33 per cent. Pickhardt, Bernhard, and Kohn<sup>11</sup> regarded 30 per cent as the maximum normal percentage of free sterol in serum. Bodansky and Bodansky<sup>2</sup> stated that the concentration of total cholesterol in plasma was between 160 and 200 mg. per 100 c.c., with the free sterol around 30 per cent with very

slight variation. On the basis of these and other studies,<sup>1, 4, 6, 10</sup> the maximum normal concentration of total cholesterol in either serum or whole blood may be regarded as 200 mg. per 100 c.c., with a maximum of 30 to 40 per cent existing as the free sterol.

TABLE II  
FREQUENCY DISTRIBUTION FOR TOTAL CHOLESTEROL

| TOTAL CHOLESTEROL<br>MG./100 C.C. | WHOLE BLOOD |         | SERUM |         | TOTAL |
|-----------------------------------|-------------|---------|-------|---------|-------|
|                                   | MALES       | FEMALES | MALES | FEMALES |       |
| 140-160                           | 2           | 2       | 0     | 0       | 4     |
| 160-180                           | 9           | 7       | 0     | 3       | 19    |
| 180-200                           | 14          | 9       | 4     | 6       | 33    |
| 200-220                           | 6           | 10      | 3     | 4       | 23    |
| 220-240                           | 8           | 2       | 5     | 5       | 20    |
| 240-260                           | 2           | 5       | 3     | 2       | 12    |
| 260-280                           | 5           | 3       | 2     | 1       | 11    |
| 280-300                           | 4           | 3       | 4     | 1       | 12    |
| 300-320                           | 4           | 0       | 1     | 4       | 9     |
| 320-340                           | 0           | 3       | 0     | 1       | 4     |
| 340-360                           | 0           | 0       | 1     | 0       | 1     |
| 360-380                           | 0           | 2       | 0     | 0       | 2     |

TABLE III  
FREQUENCY DISTRIBUTION FOR PERCENTAGE FREE CHOLESTEROL

| FREE CHOLESTEROL<br>% | WHOLE BLOOD |         | SERUM |         | TOTAL |
|-----------------------|-------------|---------|-------|---------|-------|
|                       | MALES       | FEMALES | MALES | FEMALES |       |
| 10-15                 | 1           | 1       | 0     | 0       | 2     |
| 15-20                 | 1           | 0       | 4     | 1       | 6     |
| 20-25                 | 3           | 1       | 1     | 1       | 6     |
| 25-30                 | 6           | 2       | 3     | 5       | 16    |
| 30-35                 | 5           | 2       | 5     | 2       | 14    |
| 35-40                 | 6           | 3       | 1     | 1       | 11    |
| 40-45                 | 6           | 9       | 2     | 5       | 22    |
| 45-50                 | 6           | 3       | 3     | 3       | 15    |
| 50-55                 | 7           | 9       | 2     | 4       | 22    |
| 55-60                 | 1           | 2       | 2     | 3       | 8     |
| 60-65                 | 4           | 8       | 1     | 1       | 14    |
| 65-70                 | 3           | 2       | 0     | 1       | 6     |
| 70-75                 | 2           | 3       | 0     | 0       | 5     |
| 75-80                 | 1           | 2       | 0     | 0       | 3     |

In this study, as can be seen from Tables II and III, when whole blood was used, 29 males and 28 females, or 57 per cent of the subjects investigated, had a total cholesterol concentration of more than 200 mg. per 100 c.c.; 30 males and 38 females, or 68 per cent, had a free cholesterol of more than 40 per cent. When serum was employed, 19 males and 18 females, or 74 per cent of the subjects studied, had a total cholesterol of more than 200 mg. per 100 c.c.; 10 males and 17 females, or 54 per cent, showed a free cholesterol in excess of 40 per cent.

The question now arises as to whether the comparatively high total and free cholesterol in the normal aged person has any significance. Are these increased cholesterol values a natural concomitant of old age? The persons in this study did not present any clinical evidence of liver dysfunction, biliary tract disease, diabetes, or renal impairment, which conditions are often accompanied by a hypercholesterolemia and a disturbed cholesterol partition. Arteriosclerosis is a factor that must be taken into consideration in any per-

son past 50 years of age. Kilduffe<sup>16</sup> stated that increased cholesterol values are often associated with arteriosclerosis. Leary<sup>17</sup> thought that arteriosclerosis is probably the result of abnormal cholesterol metabolism. Best and Taylor<sup>18</sup> stated that one of the means by which arteriosclerosis could be produced in rabbits was by feeding diets high in cholesterol. They also claimed that the administration of cholesterol is one of the most certain means of producing excessive deposition of neutral fats in the liver, with a resulting accumulation of cholesterol esters.

#### SUMMARY

Cholesterol studies were made in 150 normal aged persons. Of the 100 persons in whom whole blood was studied, 57 per cent had a total cholesterol concentration of more than 200 mg. per 100 c.e., and 68 per cent showed free cholesterol of more than 40 per cent. Of the 50 persons in whom serum was used, 74 per cent had a total cholesterol concentration of over 200 mg. per 100 c.e., and 54 per cent had an excess of 40 per cent free cholesterol.

We wish to thank Dr. Samuel Seidenberg for his cooperation.

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## SULFONAMIDE SOLUBILITY IN UREA\*

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SULFONAMIDE hematuria, well recognized in both clinic and laboratory, is closely associated with mechanical traumatic effects following precipitation of insoluble acetylated sulfanilyl derivatives within the urinary tract. Recent studies by Curtis and Sobin<sup>1</sup> have shown that the solubility of *acetyl-sulfapyridine* and *acetylsulfathiazole* is considerably enhanced by an alkaline pH and by increasing amounts of urea in solution. Both of these factors may well explain the difference in solubility of these substances in water and urine. The peptizing effect of urea is also seen with other more simple chemical substances.<sup>2</sup>

Analysis of the precipitated material found in the urinary tract of man and animals has shown that in sulfapyridine-treated cases more free drug is present than in the sulfathiazole-treated cases.<sup>3-6</sup> To determine a possible mechanism for the differences in free sulfonamides in uroliths, the solubility of the *free acids* of sulfapyridine, sulfathiazole, and sulfadiazine in varying concentrations of urea have been studied.

It was further hoped that the solvent effect of urea on these sulfonamides might be sufficient to allow the preparation of a soluble form for parenteral use without the attendant dangers of a high pH of the alkaline salts.

### METHOD

Urea solutions of varying concentrations from 0.1 to 10 per cent were incubated at 37° C. with an excess of sulfapyridine, sulfathiazole, and sulfadiazine, shaken at intervals, and filtered through two thicknesses of Whatman No. 42 filter paper. After appropriate dilution the free sulfonamide was determined by the method of Bratton and Marshall,<sup>7</sup> using the Evelyn colorimeter and a No. 540 filter.

### RESULTS

The results of this series of observations are shown graphically in Fig. 1. Solubility in milligrams per 100 c.c. is plotted on the ordinates, and concentration of urea in per cent on the abscissae. The order of increasing solubility is sulfadiazine, sulfapyridine, and sulfathiazole. The order of increasing solvent effect of urea is the same as their solubilities.

### DISCUSSION

It has been previously shown that the solubility of acetylsulfathiazole is slightly increased by urea, and that of acetylsulfapyridine is more definitely increased.<sup>1</sup> In neither case is the total solubility in 10 per cent urea solution above 40 mg. per 100 c.c., as contrasted with the values of 133.0 mg. per 100 c.c. for sulfathiazole and 73.3 mg. per 100 c.c. for sulfapyridine in similar 10 per cent urea solution. The difference in solubility between the free acids them-

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selves and between the free acid and their acetylated compounds is sufficient to explain the concentration of sulfapyridine and sulfathiazole in analyzed uroliths.

The solubility of sulfadiazine is quite low, and in 10 per cent urea the maximum solubility was found to be 31.5 mg. per 100 c.c. This figure approximates the solubility of acetylsulfapyridine and acetylsulfathiazole, and re-emphasizes a potential danger in the use of this drug—already experienced clinically.

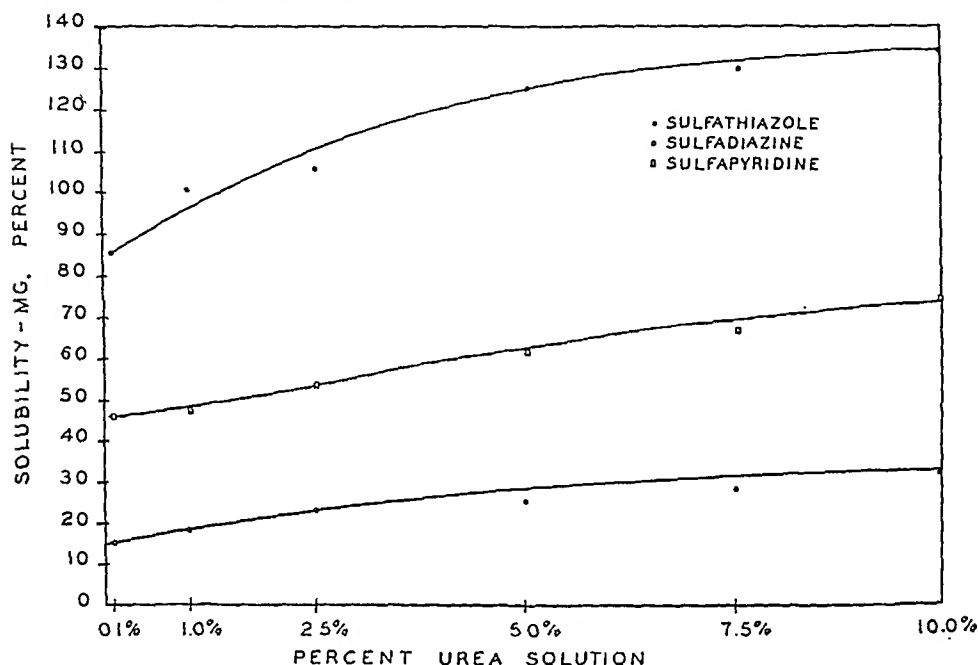


Fig. 1.

The solvent effect of urea on these sulfonamides is not of sufficient degree to be adaptable to parenteral sulfonamide therapy.

#### CONCLUSIONS

1. Urea increases the solubility of sulfathiazole, sulfapyridine, and sulfadiazine.
2. The extent of such increase in solubility may explain some related problems of renal precipitation of sulfonamides.
3. Sulfonamide's solubility in urea-sulfonamide solutions is not sufficient to warrant its use in parenteral therapy.

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# LABORATORY METHODS

## GENERAL

### A STUDY OF THE PHENOMENON OF ERYTHROCYTE SEDIMENTATION\*

#### III. ANTICOAGULATION AS A TECHNICAL SOURCE OF VARIATIONS

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IN A PREVIOUS publication a critical survey of considerable literature dealing with sedimentation was presented.<sup>1</sup> In another publication<sup>2</sup> a reliable technique for measuring sedimentation, a more practical "field technique" and evidence of the reproducibility and limitations of each were advanced. This paper includes evidence supporting my choice of the kind and amount of anticoagulant used, as well as the limitations of this choice and variations due to other kinds and quantities of anticoagulants.

The choice of a suitable means of anticoagulation for sedimentation studies in equine blood has been a perplexing problem. Many different concentrations of several kinds of anticoagulants were attempted, examples of results of which are to follow. Defibrination was also attempted but, because of varying degrees of hemolysis and somewhat variable results, was discarded. It is realized that Fahraeus<sup>3</sup> employed this method of anticoagulation quite extensively with horse blood but used as a criterion of sedimentation a single reading at the end of one hour, a method of reading which because maximum sedimentation has passed will yield somewhat comparable results in practically all samples of equine blood having fairly close volume percentages. Fahraeus admitted that his method did not in reality measure sedimentation.

In defibrinated samples sedimentation is slower than in samples with added anticoagulants. In samples in which the process of defibrination caused the least cell damage, and plasma, although darker than anticoagulant plasma, was not reddish, volume percentages were closer to those of anticoagulant samples. Even in defibrinated samples where plasma was only slightly darker and volume percentages were quite uniform, sedimentation was much slower, as for example with potassium and ammonium oxalated aliquots of the same blood sample:

| Aliquot No. | $M_z$ † | $t_{M_z}$ ‡ | $Z^S_{M_z}$ | V%   | Plasma |
|-------------|---------|-------------|-------------|------|--------|
| 1           | 26 mm.  | 10-15 min.  | 30° 20'     | 35.5 | Clear  |
| 2           | 25 mm.  | 10-15 min.  | 31° 30'     | 36   | Clear  |
| 3           | 26 mm.  | 10-15 min.  | 30° 20'     | 36-  | Clear  |

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‡Definitions:

$M_z$  = Maximum five-minute descent in millimeters of the line of demarcation between cells and plasma.

$t_{M_z}$  = Times in minutes between which  $M_z$  occurs. Since with the photographic apparatus one minute is the equivalent of 3 mm., this time-distance ratio is retained for the "field" technique.

With defibrinated aliquots of the same blood sample as the foregoing:\*

| Aliquot No. | $M_s$  | $t_{M_s}$                 | $\angle^s M_s$ | V%   | Plasma |
|-------------|--------|---------------------------|----------------|------|--------|
| 4           | 18 mm. | { 5-10 min.<br>10-15 min. | 40° 20'        | 34   | Darker |
| 5           | 19 mm. | 10-15 min.                | 38° 50'        | 33.7 | Darker |
| 6           | 20 mm. | 10-15 min.                | 37° 20'        | 33.7 | Darker |

Photographic recordings of sedimentation of aliquots of the same sample of blood, with hemolysis at a minimum, are like Fig. 1.

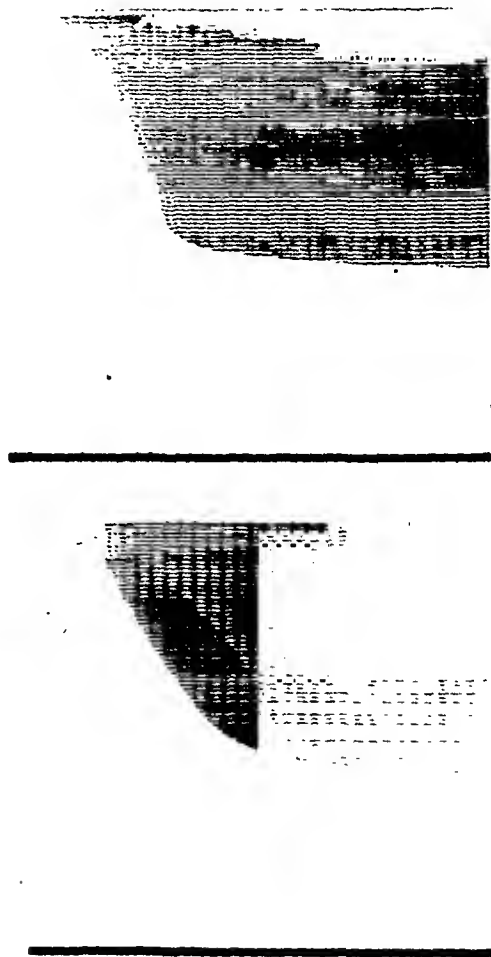


Fig. 1.—Sedimentation curves with the ammonium and potassium oxalate mixture (top) and defibrination (bottom).

\*Definitions (cont'd):

$\angle_{M_s}$  = Angle opposite the horizontal side of a right triangle having  $M_s$  as the perpendicular side and five minutes, or 15 mm., as the horizontal side.

V% = Volume per cent of packed cells.

$t_{em}$  = The perpendicular distance from the estimated point of change between the vertical flexion and horizontal flexion of the photographically recorded sigmoid curve of sedimentation of a sample and the top line of the photograph representing 100 mm. of blood column height, i.e., estimated "fall" in millimeters at the point of change.

$t_{em}$  = Time in minutes between the time of filling of the Wintrobe tube and the time that the point of change between the vertical flexion and horizontal flexion of the photographically recorded sigmoid curve of sedimentation of a sample occurs (one minute = 3 mm.)

$\angle_s$  = The least angle between the steepest slope of the sigmoid curve of sedimentation of a sample and perpendicularity.

With potassium and ammonium oxalate:

| Ex. No. | em*    | t <sub>em</sub> * | $\angle^{\circ}_g$ * | M <sub>s</sub> | tM <sub>s</sub> | $\angle^{\circ}M_s$ | V% |
|---------|--------|-------------------|----------------------|----------------|-----------------|---------------------|----|
| 1       | 31 mm. | 9 min.            | 20°                  | 30 mm.         | 5-10 min.       | 27°                 | 34 |

With defibrination:

|   |        |        |         |        |           |         |      |
|---|--------|--------|---------|--------|-----------|---------|------|
| 2 | 19 mm. | 8 min. | 29° 10' | 25 mm. | 5-10 min. | 31° 20' | 33.3 |
|---|--------|--------|---------|--------|-----------|---------|------|

It was found that, if careful measuring was employed, similar results could be obtained with similar quantities of anticoagulant. It was felt then that these anticoagulants should each be tested for the most suitable quantity and then these most suitable quantities compared. It was decided to choose that anticoagulant which had the least effect on both sedimentation and volume per cent over the widest range of concentration, and to use a minimal but safe quantity of it, realizing, of course, that there might be some individual differences in the minimal quantity.

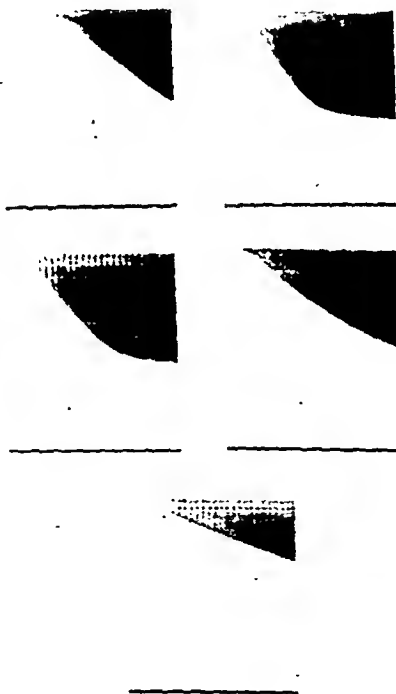


Fig. 2.—Effects of varying quantities of lithium citrate upon sedimentation.

Accurate 2 per cent solutions of the following anticoagulants were prepared in double distilled water: potassium oxalate, sodium oxalate, lithium oxalate, ammonium oxalate, potassium citrate, sodium citrate, lithium citrate, ammonium citrate, and sodium fluoride.

Also a 2 per cent solution of a mixture of 3 parts ammonium oxalate and 2 parts potassium oxalate and a 0.05 per cent solution of heparin containing 110 units per milligram were prepared.

\*See footnotes, page 1570, for definitions.

Collecting vials (0.5 ounce) previously graduated at 5 c.c. were prepared with the following concentrations of dry anticoagulants by introducing the proper quantity of the afore-mentioned solutions and allowing the water to evaporate in an incubator:

2, 4, 8, 12, 16, 20, 24 mg. for all the oxalates.

4, 8, 12, 16, 20, 24 mg. for the citrates and sodium fluoride.

5.5, 11.0, 16.5, 22.0, 27.5, 55.0 units of heparin.

Blood was collected as previously described<sup>2</sup> and divided into the 5 c.c. aliquots for each set. Blood and anticoagulant were vigorously shaken for two minutes by hand. While no specific check was made of the completeness of the mixture of anticoagulant and blood, it is felt that, since the anticoagulant was disbursed as much as possible in the collecting vial to facilitate its solution, error in this respect is probably at least minimized. Of course, previous to determination of sedimentation, each aliquot was uniformly agitated in the mechanical shaker previously described.<sup>2</sup>

Although there were minor variations in different samples, results which follow show at least trends. (In the following tabulated data *C* stands for clotting to the extent that measurements of sedimentation could not be made; *c* for evidences of small clots; *f* for what was thought to be fibrin precipitation observed in the plasma; *d* for slightly darker plasma; and *h* for plasma with a definite reddish cast.)

#### Anticoagulant—potassium oxalate:

| Mg./5 c.c. of blood | M <sub>2</sub> | t <sub>M<sub>2</sub></sub> | ∠ <sup>s</sup> M <sub>2</sub> | V%   | Plasma |
|---------------------|----------------|----------------------------|-------------------------------|------|--------|
| 2                   | C              | —                          | —                             | —    | —      |
| 4                   | 16 mm.c        | 20-25 min.                 | 43° 50'                       | 41.7 | Clear  |
| 8                   | 18 mm.         | 15-20 min.                 | 40° 20'                       | 40.3 | Clear  |
| 12                  | 17 mm.         | 15-20 min.                 | 41° 50'                       | 39.5 | Clear  |
| 16                  | 12 mm.         | 15-20 min.                 | 50° 30'                       | 38.5 | d      |
| 20                  | 6 mm.          | 10-25 min.                 | 67°                           | 37.7 | d      |

#### Anticoagulant—sodium oxalate:

| Mg./5 c.c. of blood | M <sub>2</sub> | t <sub>M<sub>2</sub></sub> | ∠ <sup>s</sup> M <sub>2</sub> | V%   | Plasma |
|---------------------|----------------|----------------------------|-------------------------------|------|--------|
| 2                   | C              | —                          | —                             | —    | —      |
| 4                   | 17 mm.         | 20-25 min.                 | 41° 50'                       | 39.5 | Clear  |
| 8                   | 16 mm.         | 15-20 min.                 | 43° 50'                       | 38   | Clear  |
| 12                  | 15 mm.         | 10-15 min.                 | 45°                           | 36.7 | Clear  |
| 16                  | 9 mm.          | 5-10 min.                  | 58° 10'                       | 36   | d      |
| 20                  | 2 mm.          | 20-25 min.                 | 81° 10'                       | 35.3 | d      |

#### Anticoagulant—lithium oxalate:

| Mg./5 c.c. of blood | M <sub>2</sub> | t <sub>M<sub>2</sub></sub> | ∠ <sup>s</sup> M <sub>2</sub> | V%   | Plasma |
|---------------------|----------------|----------------------------|-------------------------------|------|--------|
| 2                   | C              | —                          | —                             | —    | —      |
| 4                   | 15 mm.         | 15-20 min.                 | 45°                           | —    | Clear  |
| 8                   | 16 mm.         | 15-20 min.                 | 43° 50'                       | 38   | Clear  |
| 12                  | 4 mm.          | 5-25 min.                  | 74° 10'                       | 37 - | d      |
| 16                  | 2 mm.          | 20-25 min.                 | 81° 10'                       | 36.5 | d      |
| 20                  | 2 mm.          | 20-25 min.                 | 81° 10'                       | 36 + | d      |

## Anticoagulant—ammonium oxalate:

| Mg./5 c.c. of blood | $M_2$  | $t_{M_2}$  | $\angle^s M_2$ | V%   | Plasma |
|---------------------|--------|------------|----------------|------|--------|
| 2                   | C      | -          | -              | -    | -      |
| 4                   | 17 mm. | 15-20 min. | 41° 50'        | 39.3 | Clear  |
| 8                   | 18 mm. | 15-20 min. | 40° 20'        | 40.5 | Clear  |
| 12                  | 17 mm. | 20-25 min. | 41° 50'        | 41   | Clear  |
| 16                  | 17 mm. | 20-25 min. | 41° 50'        | 42.3 | Clear  |
| 20                  | 17 mm. | 25-30 min. | 41° 50'        | 43   | Clear  |

## Anticoagulant—potassium citrate:

| Mg./5 c.c. of blood | $M_2$  | $t_{M_2}$  | $\angle^s M_2$ | V%   | Plasma |
|---------------------|--------|------------|----------------|------|--------|
| 4                   | C      | -          | -              | -    | -      |
| 8                   | C      | -          | -              | -    | -      |
| 12                  | C      | -          | -              | -    | -      |
| 16                  | 17 mm. | 15-20 min. | 41° 50'        | 36.5 | Clear  |
| 20                  | 16 mm. | 10-20 min. | 43° 50'        | 36.3 | Clear  |
| 24                  | 15 mm. | 15-20 min. | 45°            | 36   | Clear  |

## Anticoagulant—sodium citrate:

| Mg./5 c.c. of blood | $M_2$   | $t_{M_2}$  | $\angle^s M_2$ | V%   | Plasma |
|---------------------|---------|------------|----------------|------|--------|
| 4                   | C       | -          | -              | -    | -      |
| 8                   | C       | -          | -              | -    | -      |
| 12                  | 19 mm.c | 10-15 min. | 38° 40'        | 37.3 | Clear  |
| 16                  | 18 mm.  | 10-15 min. | 40° 20'        | 36.5 | Clear  |
| 20                  | 13 mm.  | 15-25 min. | 48° 30'        | 35.7 | d      |
| 24                  | 7 mm.   | 5-10 min.  | 64° 10'        | 34.5 | d      |

## Anticoagulant—lithium citrate:

| Mg./5 c.c. of blood | $M_2$  | $t_{M_2}$  | $\angle^s M_2$ | V%   | Plasma |
|---------------------|--------|------------|----------------|------|--------|
| 4                   | C      | -          | -              | -    | -      |
| 8                   | 10 mm. | 15-20 min. | 50° 30'        | 37.7 | Clear  |
| 12                  | 18 mm. | 10-15 min. | 40° 20'        | 36.5 | Clear  |
| 16                  | 17 mm. | 10-15 min. | 41° 50'        | 34.7 | Clear  |
| 20                  | 16 mm. | 10-15 min. | 42° 50'        | 34   | Clear  |
| 24                  | 10 mm. | 10-15 min. | 55° 30'        | 33   | d      |

## Anticoagulant—ammonium citrate:

| Mg./5 c.c. of blood | $M_2$   | $t_{M_2}$  | $\angle^s M_2$ | V%   | Plasma |
|---------------------|---------|------------|----------------|------|--------|
| 4                   | C       | -          | -              | -    | -      |
| 8                   | 23 mm.c | 15-20 min. | 33° 30'        | 41   | Clear  |
| 12                  | 21 mm.  | 15-20 min. | 35° 50'        | 41.3 | Clear  |
| 16                  | 22 mm.  | 15-20 min. | 34° 40'        | 41.8 | Clear  |
| 20                  | 21 mm.  | 10-15 min. | 35° 50'        | 42.3 | Clear  |
| 24                  | 20 mm.  | 10-15 min. | 37° 10'        | 42.8 | Clear  |

## Anticoagulant—sodium fluoride:

| Mg./5 c.c. of blood | $M_2$ | $t_{M_2}$   | $\angle^s M_2$ | V%   | Plasma |
|---------------------|-------|-------------|----------------|------|--------|
| 4                   | C     | -           | -              | -    | -      |
| 8                   | C     | -           | -              | -    | -      |
| 12                  | 3 mm. | 10-35 min.  | 77° 50'        | 34.0 | Clear  |
| 16                  | 2 mm. | 40-45 min.  | 81° 10'        | 33.5 | Clear  |
| 20                  | 1 mm. | 10-60+ min. | 85° 10'        | 33   | Clear  |

## Antieoagulant—ammonium and potassium oxalate mixture:

| Mg./5 c.c. of blood | $M_1$  | $t_{M_2}$                      | $\angle^s M_2$ | V%   | Plasma |
|---------------------|--------|--------------------------------|----------------|------|--------|
| 2                   | C      | -                              | -              | -    | -      |
| 4                   |        | (Compared in later experiment) |                |      |        |
| 8                   | 20 mm. | 20-25 min.                     | 37° 10'        | 37.7 | Clear  |
| 12                  | 19 mm. | 10-15 min.                     | 38° 40'        | 38.3 | Clear  |
| 16                  | 19 mm. | 15-20 min.                     | 38° 40'        | 38   | Clear  |
| 20                  | 18 mm. | 15-20 min.                     | 40° 20'        | 37.7 | Clear  |
| 24                  | 17 mm. | 10-15 min.                     | 41° 50'        | 37.5 | d      |

## Antieoagulant—heparin:

| Units/5 c.c. of blood | $M_1$    | $t_{M_2}$  | $\angle^s M_2$ | V%   | Plasma |
|-----------------------|----------|------------|----------------|------|--------|
| 5.5                   | C        | -          | -              | -    | -      |
| 11.0                  | 15 mm.c  | 15-20 min. | 45°            | 42 + | Clear  |
| 16.5                  | 19f      | 15-20 min. | 38° 40'        | 42 + | Clear  |
| 22.0                  | 18f      | 15-20 min. | 40° 20'        | 42.7 | Clear  |
| 27.5                  | 20 mm.   | 15-20 min. | 37° 10'        | 42.7 | Clear  |
| 55.0                  | 16.5 mm. | 10-15 min. | 42° 50'        | 42.7 | Clear  |

## Antieoagulant—heparin. Repeated on same aliquots:

| Units/5 c.c. of blood | $M_1$     | $t_{M_2}$  | $\angle^s M_2$ |
|-----------------------|-----------|------------|----------------|
| 5.5                   | C         | -          | -              |
| 11.0                  | C         | -          | -              |
| 16.5                  | 19.5 mm.f | 15-20 min. | 38°            |
| 22.0                  | 20.5 mm.  | 10-15 min. | 36° 40'        |
| 27.5                  | 19.5 mm.  | 10-15 min. | 38°            |
| 55.0                  | 16.5 mm.  | 15-20 min. | 42° 50'        |

From the foregoing data one can see that, in general, sedimentation tends to decrease the more antieoagulant is used. Volume percentages tend to decrease with potassium, sodium, and lithium oxalates and citrates as the amount of antieoagulant is increased. With ammonium oxalate and ammonium citrate the reverse is true. With heparin and the potassium and ammonium oxalate mixture volume percentages tend to remain fairly close. With the ammonium salts, the potassium and ammonium oxalate mixture and heparin, sedimentation values tend to remain close over a wider range of concentrations than with the others. Since the volume percentages with ammonium oxalate and ammonium citrate vary considerably with concentration, they may be considered as less efficient than heparin and the potassium and ammonium oxalate mixture.

Sodium fluoride apparently cannot be used for equine blood.

Although heparin has many good features as an antieoagulant, it also has some which are disappointing. In practice it is less dependable than the potassium and ammonium oxalate mixture, there apparently being a wide variation in the minimal quantity necessary for antieoagulation. With some horse's blood as low as 11 units per 5 c.c. is sufficient for apparently complete antieoagulation, whereas with the blood of others three or four times that amount sometimes is not. Regardless of whether this is the result of poor solubility or not, or whether it is inherent in the action of the preparation itself or not, such variation seems to exist and naturally leads one to be a bit dubious of the results he obtains. The appearance of the plasma often is an aid in judging the degree of antieoagulation, since in equine blood with heparin the degree of what is apparently lipemia

is less than with the other anticoagulants. Any cloudiness of the plasma, where there are particles of various sizes, or a "flaky" appearance, is thought to suggest incomplete anticoagulation, especially since this phenomenon occurs only at concentrations nearest to clotting.

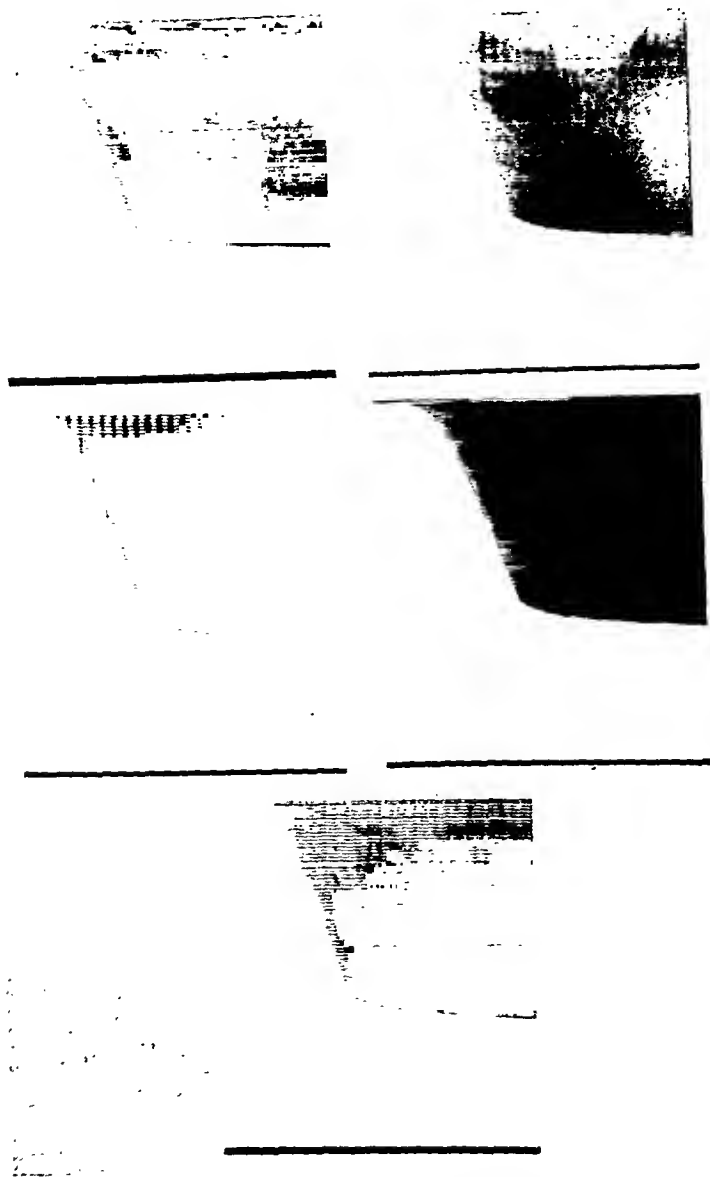


Fig. 3.—Effects of varying quantities of ammonium oxalate upon sedimentation.

In practice the ammonium and potassium oxalate mixture has been most satisfactory.

As photographic examples of the effects of those anticoagulants which decrease volume per cent as well as sedimentation consider the photographs in Fig. 2.

## Anticoagulant—lithium citrate:

| Ex. No. | Mg./5 c.c. blood | em     | $t_{em}$ | $\angle^s_g$ |  |
|---------|------------------|--------|----------|--------------|--|
| 1       | 8                | 19 mm. | 16 min.  | 48° 20'      |  |
| 2       | 12               | 20 mm. | 8.3 min. | 31° 10'      |  |
| 3       | 16               | 18 mm. | 8.2 min. | 36°          |  |
| 4       | 20               | 10 mm. | 7.3 min. | 47° 50'      |  |
| 5       | 24               | 7 mm.  | 7.2 min. | 62° 20'      |  |

| Ex. No. | $M_s$    | $t_{M_s}$  | $\angle^s_{M_s}$ | V%   | Plasma |
|---------|----------|------------|------------------|------|--------|
| 1       | 13 mm.   | 15-20 min. | 48° 20'          | 37.7 | Clear  |
| 2       | 20 mm.   | 5-10 min.  | 37° 20'          | 36.5 | Clear  |
| 3       | 19 mm.   | 5-10 min.  | 38° 40'          | 34.7 | Clear  |
| 4       | 12.5 mm. | 5-10 min.  | 49° 40'          | 34   | Clear  |
| 5       | 7 mm.    | 5-10 min.  | 64°              | 33   | d      |

As an example of those anticoagulants which increase volume percentage (Fig. 3) :

## Anticoagulant—ammonium oxalate:

| Ex. No. | Mg./5 c.c. blood | em     | $t_{em}$  | $\angle^s_g$ |  |
|---------|------------------|--------|-----------|--------------|--|
| 1       | 4                | 31 mm. | 9 min.    | 20° 20'      |  |
| 2       | 8                | 29 mm. | 10.7 min. | 16° 50'      |  |
| 3       | 12               | 24 mm. | 8.3 min.  | 16° 20'      |  |
| 4       | 16               | 29 mm. | 9.3 min.  | 17° 10'      |  |
| 5       | 20               | 33 mm. | 9.5 min.  | 18° 00'      |  |

| Ex. No. | $M_s$    | $t_{M_s}$  | $\angle^s_{M_s}$ | V%   | Plasma |
|---------|----------|------------|------------------|------|--------|
| 1       | 34 mm.   | 5-10 min.  | 24° 00'          | 28.7 | Clear  |
| 2       | 34.5 mm. | 10-15 min. | 23° 40'          | 28.7 | Clear  |
| 3       | 37 mm.   | 5-10 min.  | 22° 10'          | 29.3 | Clear  |
| 4       | 31 mm.   | 5-10 min.  | 26°              | 30 + | Clear  |
| 5       | 33 mm.   | 5-10 min.  | 24° 40'          | 30.3 | Clear  |

As an example of heparin (Fig. 4) :

| Ex. No. | Units/5 c.c. blood | em       | $t_{em}$  | $\angle^s_g$ |  |
|---------|--------------------|----------|-----------|--------------|--|
| 1       | 11                 | 17.5 mm. | 10.5 min. | 32° 40'      |  |
| 2       | 16.5               | 22.5 mm. | 9.2 min.  | 24° 30'      |  |
| 3       | 22                 | 25.0 mm. | 9.0 min.  | 23° 30'      |  |
| 4       | 27.5               | 23.0 mm. | 8.8 min.  | 24° 10'      |  |
| 5       | 55                 | 22.0 mm. | 10.5 min. | 23° 50'      |  |

| Ex. No. | $M_s$  | $t_{M_s}$  | $\angle^s_{M_s}$ | V%   | Plasma |
|---------|--------|------------|------------------|------|--------|
| 1       | 20 mm. | 10-15 min. | 37° 30'          | 42 + | f      |
| 2       | 25 mm. | 5-10 min.  | 31° 20'          | 42 + | Clear  |
| 3       | 27 mm. | 5-10 min.  | 29° 20'          | 42.7 | Clear  |
| 4       | 25 mm. | 5-10 min.  | 31° 20'          | 42.7 | Clear  |
| 5       | 23 mm. | 5-10 min.  | 33° 20'          | 42.7 | Clear  |

Fig. 5 represents peculiar curves where evidence of incomplete anticoagulation exists. Needless to say, measurements would be deceiving.

To continue, *apparent minimal quantities* of the various anticoagulants were compared on the same sample of blood with results similar to the following:

Sample No. 1:

| Aliquot No. | Anticoagulants                 | Mg./5 c.c. blood | $M_s$  | $t_{M_s}$  | $\angle^s_{M_s}$ | V%   |
|-------------|--------------------------------|------------------|--------|------------|------------------|------|
| 1           | Ammonium and potassium oxalate | 10 mg.           | 18 mm. | 20-25 min. | 40° 10'          | 40   |
| 2           | Potassium oxalate              | 8 mg.            | 19 mm. | 10-15 min. | 39° 50'          | 35.7 |
| 3           | Ammonium oxalate               | 8 mg.            | 18 mm. | 15-20 min. | 40° 10'          | 42.3 |
| 4           | Heparin                        | 15 units         | 21 mm. | 10-15 min. | 36°              | 38.7 |





Fig. 4.—Effects of varying quantities of heparin upon sedimentation.

*Sample No. 2:*

| Aliquot No. | Anticoagulants                      | Mg./5 c.c.<br>blood | $M_s$  | $t_{M_s}$  | $\angle^s M_s$ | V%   |
|-------------|-------------------------------------|---------------------|--------|------------|----------------|------|
| 1           | Ammonium and potas-<br>sium oxalate | 10 mg.              | 15 mm. | 15-20 min. | 45°            | 42.5 |
| 2           | Lithium oxalate                     | 8 mg.               | 15 mm. | 15-20 min. | 45°            | 39   |
| 3           | Lithium citrate                     | 12 mg.              | 15 mm. | 15-20 min. | 45°            | 39.7 |
| 4           | Heparin                             | 15 units            | 15 mm. | 20-25 min. | 45°            | 42.3 |

*Sample No. 3:*

| Aliquot No. | Anticoagulants                 | Mg./5 c.c.<br>blood | $M_s$    | $t_{M_s}$  | $\angle^s M_s$ | V%   |
|-------------|--------------------------------|---------------------|----------|------------|----------------|------|
| 1           | Ammonium and potassium oxalate | 10 mg.              | 21 mm.   | 15-20 min. | 36°            | 38.5 |
| 2           | Potassium oxalate              | 16 mg.              | 23 mm.   | 10-15 min. | 33° 40'        | 35.5 |
| 3           | Sodium fluoride                | 12 mg.              | 2 mm.    | 10-15 min. | 82° 10'        | 32.5 |
| 4           | Heparin                        | 15 units            | 24.5 mm. | 10-15 min. | 31° 50'        | 38.5 |

*Sample No. 4:*

| Aliquot No. | Anticoagulants                 | Mg./5 c.c.<br>blood | $M_s$  | $t_{M_s}$  | $\angle^s M_s$ | V% |
|-------------|--------------------------------|---------------------|--------|------------|----------------|----|
| 1           | Ammonium and potassium oxalate | 10 mg.              | 19 mm. | 20-25 min. | 39° 50'        | 43 |
| 2           | Ammonium and potassium oxalate | 4 mg.               | 20 mm. | 15-20 min. | 37° 10'        | 42 |
| 3           | Sodium oxalate                 | 8 mg.               | 19 mm. | 10-15 min. | 39° 20'        | 44 |
| 4           | Ammonium citrate               | 12 mg.              | C      | -          | -              | -  |

*Sample No. 5:*

| Aliquot No. | Anticoagulants                 | Mg./5 c.c.<br>blood | $M_s$    | $t_{M_s}$  | $\angle^s M_s$ | V%   |
|-------------|--------------------------------|---------------------|----------|------------|----------------|------|
| 1           | Ammonium and potassium oxalate | 10 mg.              | 20.5 mm. | 15-20 min. | 36° 40'        | 41.5 |
| 2           | Sodium citrate                 | 12 mg.              | C        | -          | -              | -    |
| 3           | Sodium citrate                 | 16 mg.              | 20 mm.   | 15-20 min. | 37° 10'        | 43.3 |
| 4           | Ammonium citrate               | 15 mg.              | 15.5 mm. | 15-20 min. | 44° 10'        | 46 - |

*Sample No. 6:*

| Aliquot No. | Anticoagulants                 | Mg./5 c.c.<br>blood | $M_s$    | $t_{M_s}$  | $\angle^s M_s$ | V%   |
|-------------|--------------------------------|---------------------|----------|------------|----------------|------|
| 1           | Ammonium and potassium oxalate | 10 mg.              | 17 mm.   | 25-30 min. | 41° 40'        | 45 + |
| 2           | Ammonium and potassium oxalate | 4 mg.               | 17.5 mm. | 15-20 min. | 41°            | 45 - |
| 3           | Ammonium and potassium oxalate | 2 mg.               | C        | -          | -              | -    |
| 4           | Sodium oxalate                 | 8 mg.               | 18 mm.   | 15-20 min. | 40° 10'        | 41.5 |

The amount of heparin used was found by previous trial to be sufficient for each particular experiment, at least as far as general appearance was concerned. Wherever any signs of clotting were apparent, or even strongly suspected, the amount of the particular anticoagulant used was increased, and the experiment was repeated. In practice it was found that even with blood from one and the same animal variations in the minimal quantity of anticoagulant occurred from time to time—this being true in both pathologic and apparently normal samples, even though perhaps more true in the pathologic blood. Realizing, of course, that large errors do occur in attempting to measure as small quantities of anticoagulants as were here used, and realizing that measuring exact small quantities of blood is also subject to large error, nevertheless in spite of these I feel that minimal quantities vary in the same animal, normal or not.

As an interesting adjunct to this problem various dilutions of these various anticoagulants were prepared and the *resistance of red blood cells* was tested in them. Results as listed are quite obvious (*h* signifies complete hemolysis; *p*, partial hemolysis; *s*, suspicious; and *o*, no hemolysis).

|                           | Immediately | 1 hour |
|---------------------------|-------------|--------|
| Potassium oxalate (%):    |             |        |
| 0.2                       | h           | h      |
| 0.4                       | h           | h      |
| 0.6                       | h           | h      |
| 0.8                       | p           | p      |
| 1.0                       | o           | o      |
| 1.2                       | o           | o      |
| Sodium oxalate (%):       |             |        |
| 0.2                       | h           | h      |
| 0.4                       | h           | h      |
| 0.6                       | s           | p      |
| 0.8                       | o           | o      |
| 1.0                       | o           | o      |
| 1.2                       | o           | o      |
| Lithium oxalate (%):      |             |        |
| 0.2                       | h           | h      |
| 0.4                       | h           | h      |
| 0.6                       | o           | o      |
| 0.8                       | o           | o      |
| 1.0                       | o           | o      |
| 1.2                       | o           | o      |
| Ammonium oxalate (%):     |             |        |
| 0.4                       | h           | h      |
| 0.6                       | h           | h      |
| 0.8                       | h           | h      |
| 1.0                       | h           | h      |
| 2.0                       | h           | h      |
| Half saturated (about 4%) | p           | h      |
| Saturated (about 8%)      | s           | h      |
| Potassium citrate (%):    |             |        |
| 0.2                       | h           | h      |
| 0.4                       | h           | h      |
| 0.6                       | h           | h      |
| 0.8                       | h           | h      |
| 1.0                       | s           | p      |
| 1.2                       | o           | o      |
| 1.4                       | o           | o      |
| Sodium citrate (%):       |             |        |
| 0.2                       | h           | h      |
| 0.4                       | h           | h      |
| 0.6                       | h           | h      |
| 0.8                       | h           | h      |
| 1.0                       | p           | h      |
| 1.2                       | o           | p      |
| 1.4                       | o           | o      |
| Lithium citrate (%):      |             |        |
| 0.2                       | h           | h      |
| 0.4                       | h           | h      |
| 0.6                       | h           | h      |
| 0.8                       | h           | h      |
| 1.0                       | s           | h      |
| 1.2                       | o           | o      |
| Sodium fluoride (%):      |             |        |
| 0.2                       | h           | h      |
| 0.4                       | s           | p      |
| 0.6                       | o           | o      |
| 0.8                       | o           | o      |
| 1.0                       | o           | o      |
| Ammonium citrate (%):     | Immediately | 1 hour |
| 0.2                       | h           | h      |
| 0.4                       | p           | p      |
| 0.6                       | s           | s      |
| 0.8                       | o           | s      |
| 1.0                       | o           | p      |
| 1.2                       | o           | p      |
| 1.4                       | o           | p      |
| 1.6                       | o           | o      |
| 1.8                       | o           | o      |
| 2.0                       | o           | o      |
| 5.0                       | o           | o      |
| 10.0                      | o           | o      |

Four parts of ammonium oxalate and 6 parts of potassium oxalate:

| Solution (%): | Immediately | 1 hour |
|---------------|-------------|--------|
| 0.2           | h           | h      |
| 0.4           | h           | h      |
| 0.6           | s           | h      |
| 0.8           | s           | h      |
| 1.0           | s           | h      |
| 1.2           | s           | p      |
| 1.4           | o           | p      |
| 1.6           | o           | o      |
| 1.8           | o           | o      |
| 2.0           | o           | o      |

Equal parts of ammonium and potassium oxalate:

| Solution (%): | Immediately | 1 hour |
|---------------|-------------|--------|
| 0.2           | h           | h      |
| 0.4           | p           | p      |
| 0.6           | o           | p      |
| 0.8           | o           | s      |
| 1.0           | o           | s      |
| 1.2           | o           | o      |
| 1.4           | o           | o      |
| 1.6           | o           | o      |
| 1.8           | o           | o      |
| 2.0           | o           | o      |

Six parts of ammonium oxalate and 4 parts of potassium oxalate:

| Solution (%): | Immediately | 1 hour |
|---------------|-------------|--------|
| 0.2           | h           | h      |
| 0.4           | h           | h      |
| 0.6           | s           | h      |
| 0.8           | s           | p      |
| 1.0           | s           | p      |
| 1.2           | o           | p      |
| 1.4           | o           | p      |
| 1.6           | o           | o      |
| 1.8           | o           | o      |
| 2.0           | o           | o      |

Consideration of the foregoing data is interesting in that the point of beginning hemolysis in all salts except one was in solutions of less than 2 per cent. In the oxalates, with the exception of ammonium oxalate, and in sodium fluoride beginning hemolysis was in solutions of less than 1 per cent, while in the citrates, including ammonium citrate, it began in solutions between 1 and 2 per cent. All dilutions of ammonium oxalate hemolyzed the red blood cells, although a saturated solution (approximately 8 per cent) offered some protection for a fraction of an hour.

Would it be incorrect to say that oxalates except ammonium oxalate offer greater protection against cell damage than the citrates?

The reaction of ammonium oxalate is indeed an interesting one, since in all dilutions up to saturation hemolysis occurs when 1 drop of fresh blood is added to 2 c.c. of the solution. No doubt, when used alone as an anticoagulant, the quantity necessary (about 8 to 12 mg.) for anticoagulation is so small in proportion to the amount of blood used (5 c.c.) that clinically at least evidence of hemolysis is not observed. The quantity necessary for the ammonium and potassium oxalate mixture is even less (6 mg.). Another factor must be considered, namely, the possibility that potassium oxalate may somewhat protect the red blood cells against the hemolyzing effects of ammonium oxalate. Concerning

the proportion of these two salts it may be said that a slight excess of ammonium oxalate (which according to previous observations swells the cells, as evidenced by volume percentages greater than those of heparinized samples) seems necessary to overcome the shrinkage effect of potassium oxalate, such shrinkage being evidenced by volume percentages less than those of heparinized samples.

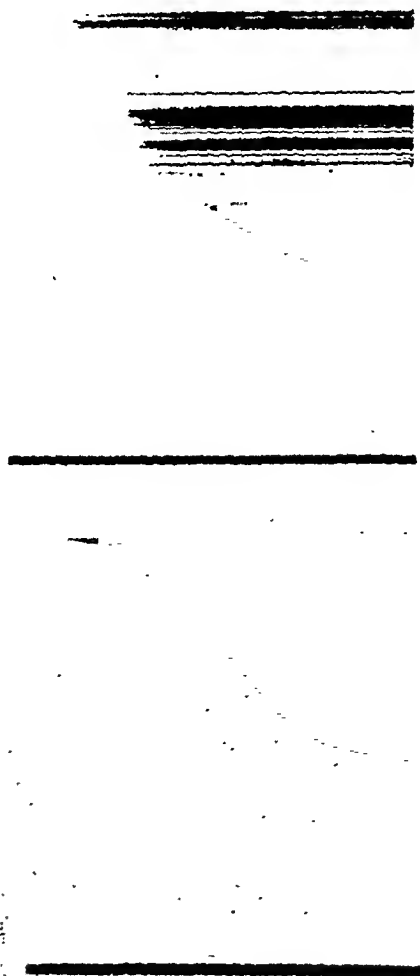


Fig. 5.—Sedimentation curves of samples with incomplete anticoagulation.

A proportion of 6 parts of ammonium to 4 parts of potassium oxalate seems to produce volume percentages closer to those of heparinized samples than other proportions or even other single salts.

In practice, between 4 and 12 mg. of the mixture of ammonium and potassium oxalate per 5 c.c. of blood have been generally considered safe. Ten milligrams per 5 c.c. as a routine quantity so far has been satisfactory. No evidence of clot has been detected in any sample as yet; photographed curves have been smooth and not irregular, as sometimes occurs with heparin. Hemolysis has not

occurred in any normal sample, and in the few pathologic samples where it has occurred, it has also occurred with heparin, and volume percentages of replicate samples as well as curves of sedimentation have been very close.

The fourth and last publication of this series will include a discussion of the effects of variations in venous stasis, delay, temperature of sample, containers, agitation, and quantity of formed elements upon the measurement of sedimentation.

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## A SHAKING MACHINE AND CONTAINERS FOR SHAKING SPUTUMS AND OTHER BODY FLUIDS DURING THE PROCESS OF HOMOGENIZATION\*

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**L**ITTLE question can be raised that marked agitation of tuberculous sputum during homogenization breaks up tenacious matter and small caseous or pussy particles, and increases the distribution of the tubercle bacilli in the specimen, thereby making possible a more accurate count of bacilli by microscopic examination of the smear.

There are many types of agitating and shaking machines from which to choose, but from the standpoint of performance and durability, the Red Devil,† or a similar type, is the most satisfactory. The model described in this article will shake a can of paint of any size up to a gallon, and the rate of agitation is 760 three-dimensional shakes per minute.

For our work a special container had to be designed for holding more than one specimen bottle in place during agitation. Also, it had to be so constructed that the bottles or cans could be placed inside the container in a way that would allow for differences in height, size, and shape, and would keep stoppers or lids firmly in place during the shaking operation.

#### DESCRIPTION OF CONTAINER

Fig. 1 shows a vertical section taken on the center line through the large group container, and shows vertical sections through two of the individual containers.

Fig. 2 is a plan view looking down on the group container with the top section (1) removed, and shows the arrangement of the individual containers within the group container.

\*From the Research and Clinical Laboratory, Trudeau Sanatorium, Trudeau.

†Made by Landon P. Smith, Irvington, N. Y.

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Fig. 3 shows a vertical section taken on the center line through an individual container.

Fig. 4 is a plan view looking down with the top section (1) removed, and shows the specimen container in place.

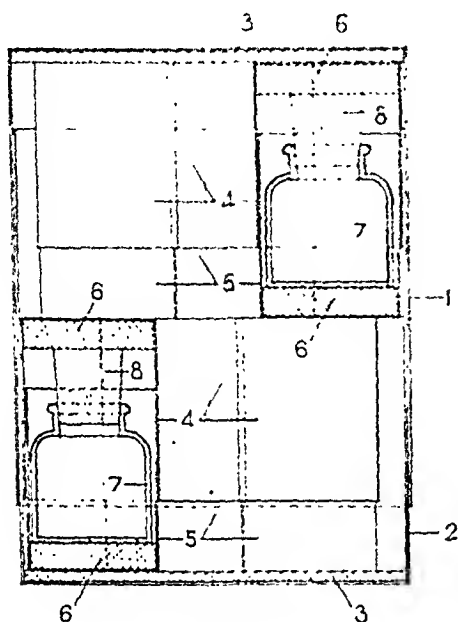


FIG. 4

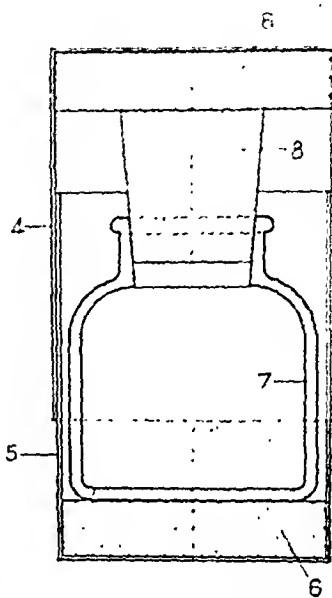


FIG. 3

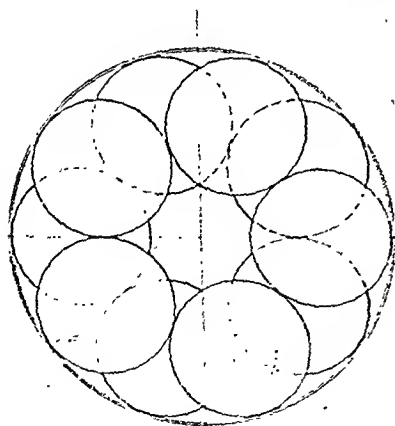


FIG. 2

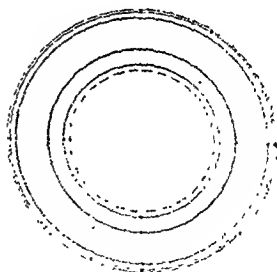
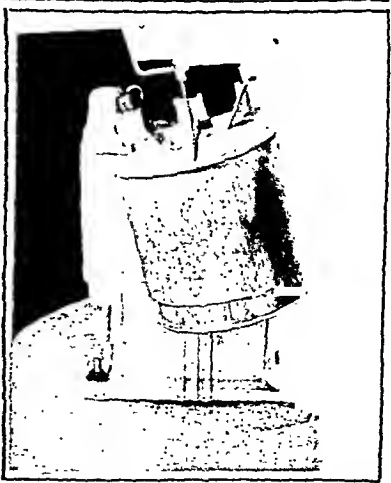
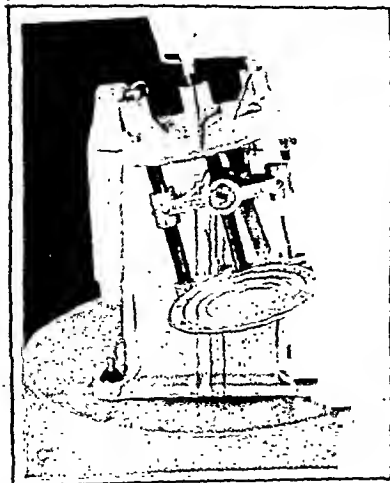
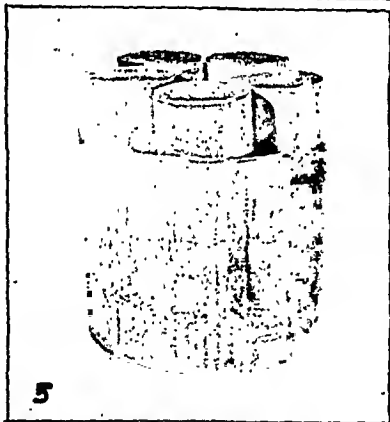
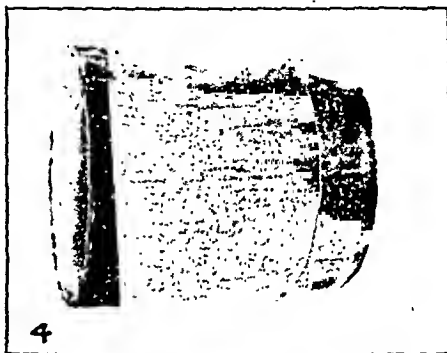
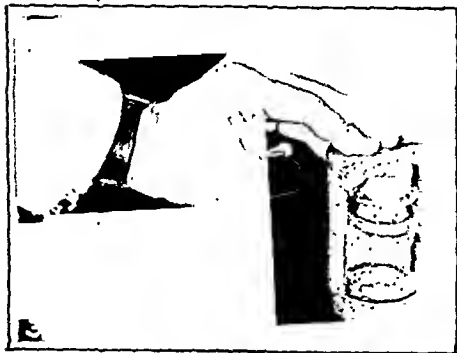
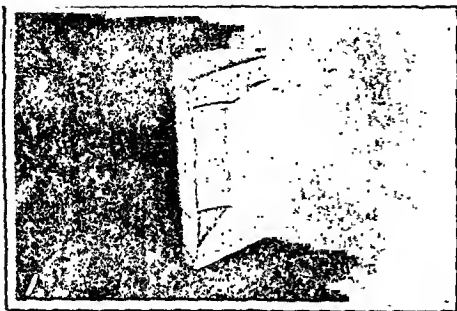


FIG. 1

The group container consists of two sections, the top section (1) fitting over the bottom section (2). It is designed to hold a given number of smaller indi-





vidual containers. The size of the group container is determined by the size and number of individual containers to be accommodated.

A cushion of soft rubber, or some other resilient material (3), is placed inside each section, as shown, to allow for variation in height of individual containers and to prevent movement of such containers during the shaking operation.

The individual container consists of two sections, the top section (4) fitting partly or completely over the bottom section (5), and it is designed to hold a specimen container (7). A rubber sponge cushion (6) is placed in each section, as shown, to compensate for the variation in height of stopper (8), and to provide for resiliency during the shaking process.

Plate II shows the following:

- 1, A cross section of an individual container.
- 2, A cross section of an individual container with bottle in place.
- 3, A cross section of an individual container with bottle in place under pressure. Note rubber compression.
- 4, A group container.
- 5, A group container with lid removed. Note also the upper layer of individual containers.
- 6, Shows how the individual containers stack in the group container. Also, a cross section of an individual container with bottle in place.
- 7, The shaking machine mounted on a concrete base which, in turn, is mounted on four rubber sponge cushions.
- 8, Shaking machine with group container, with individual container inside fastened in clamp and ready for shaking.

Our experience has proved that the use of this shaking machine with its specially designed containers, results in a better homogenization of sputum. The concentration of sodium hydroxide can be reduced. Those specimens that usually require 4 per cent routinely, can be digested with 3 per cent, and with as little as 2 per cent, depending upon the appearance of the sputum, thus decreasing the loss of acid fastness, and lessening the chance of killing the tubercle bacilli by the digesting mixture.

The marked agitation and speed of the shaking process make possible a more even distribution of the tubercle bacilli, and this enables us to obtain an accurate count of bacilli in concentrated smear, especially in the Gaffky I and the so-called negative specimens. By this method of shaking we have raised our positive findings 11.0 per cent, particularly in the low Gaffky counts (I, II, and III).

# A SIMPLE SURGICAL TECHNIQUE FOR DEVIATING THE ENTIRE URINE INTO THE BLOOD STREAM\*

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IN CONNECTION with many problems of renal physiology it is of great importance to establish whether a change elicited by damage to renal tissue is caused by interference with the excretory functions of the kidney or whether it is due to a derangement of its endocrine and metabolic activities. In such instances the technique usually employed is to compare the results of complete nephrectomy with those caused by ligation of both ureters. It is then assumed that while nephrectomy eliminates all functions of the kidney, ligation of the ureters abolishes only renal excretion. It has often been emphasized, however, that after ligation of the ureters some urine is still produced so that hydro-nephrosis results. Hence this technique does not eliminate excretion completely. Furthermore, the often very high pressure which develops in the renal pelvis is transmitted to the kidney cells and damages them severely, so that not only the excretory, but also all the renal functions must suffer to some extent. It should also be borne in mind that a kidney, whose excretory function is gradually abolished by back pressure, is not normal, and that inhibition of excretory work may in itself interfere with other activities of the kidney cells.

The object of this study is to describe an experimental technique which makes it possible to eliminate the result of renal excretion completely, without actually interfering with the process of urine production or with any other kidney function. The fundamental principle of this method is to prevent urine elimination by ligation of the urethra, and then to establish a communication between the bladder and the vena cava. Thus the urine which continues to be formed and accumulates in the bladder is led back into the blood. The technique is readily adaptable to most laboratory animals, but up to the present it has mainly been used for experiments on the rat.

The procedure is as follows: The rat is anesthetized with ether and tied on its back to an operating board. An incision is made through the skin and muscle layers in the midline from the pubic region to the umbilicus. The intestines are pushed cranial and the rectum is pushed to the left by means of cotton pads which can be held in position by a retractor whose other end is hooked onto some heavy object on the table. This arrangement makes it possible to obtain perfect exposure of the vena cava between the pelvis and the kidney region without the help of an assistant. Now the peritoneal covering of the posterior abdominal wall is severed by mere traction between two fingers, and the vena cava

\*From the Department of Anatomy, McGill University, Montreal.  
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is carefully separated from its surroundings between its bifurcation into the two iliac veins and the right iliohypogastric vein. This is accomplished by blunt dissection using two curved forceps, each of which holds a small cotton pad. With the help of these pads it is easy to crush and sever the numerous little venules which enter the cava from the psoas major and the ventral surface of the lumbar vertebrae. Some of these latter venules are very large, yet they can all be severed without ligature if they are first crushed with the pads. If they tend to bleed, the hemorrhage is readily stopped by pressing a minute piece of cotton on them until clotting occurs. After this the vena cava is completely occluded by a ligature placed above the junction of the iliac veins. Another ligature is laid loosely around the cava just underneath the iliohypogastric vein. It should be emphasized here that in the rat ligature of the vena cava caudad from the renal veins produces no symptoms as there is an adequate collateral circulation.



Fig. 1.—First stage of the operation. The bladder is exposed and the blunt end of the curved cannula is tied into its cranial end with the ligature shown by arrow A. This ligature fits into one groove of the cannula, while the other groove is visible between the pointed end and the anatomical forceps which holds the cannula. Arrow B points to the ligature occluding the urethra. A curved forceps holds this ligature.

Now the urinary bladder is pulled forward and a ligature is tied around the urethra just caudad from the point where the ureters enter the bladder. In order to maintain a good blood supply to the bladder it is well to avoid any interference with the large veins which accompany the ureters. After loss of urine has thus been prevented by occluding the urethra, a small incision is made on the apex of the bladder, and the blunt end of a steel cannula is introduced into the lumen. This cannula may be fastened in this position by a single ligature, as shown in Fig. 1.

The cannula, which will connect the bladder with the vena cava, is made from gauge 16 or gauge 13 injection needles by cutting off about 1 to 1½ inches

near the pointed end, making one circular groove near the blunt and one near the pointed end to hold ligatures in place. It is well to bend the cannula slightly, as shown in Figs. 1 and 2, so as to adapt them better to the anatomy of the pelvic region. In order to prevent clotting it is advisable always to use the widest gauge that can be introduced into the vena cava. If this precaution is observed, and the inner surface of the needle is heparinized, thrombi do not form, and the passage of urine into the vein proceeds readily throughout the experiment. Reflux of blood from the vein into the bladder is rarely seen, since the pressure is usually greater in the bladder than it is in the vein. Both in order to avoid reflux and as a precaution against a delay in the re-entrance of urine into the blood, most of the bladder wall should be tied off with the ligature which fastens the cannula into the bladder. Only a small stump with the ureteral orifices needs to be left intact.

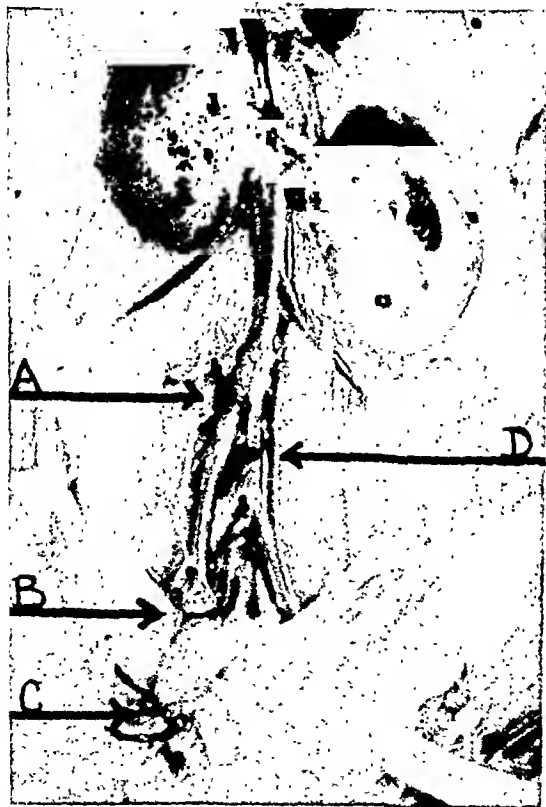


Fig. 2.—Dissection of the lower abdomen showing the position of the cannula after completion of the second stage of the operation. Arrow A designates the ligature which ties the vena cava to the pointed end of the cannula which is now inserted into the lumen of the vein. Arrow B points to the ligature fastening the blunt end of the cannula into the cranial end of the bladder. Arrow C points to the ligature occluding the urethra. Arrow D indicates the ligature occluding the vena cava above its bifurcation into the iliac veins.

The bladder is now washed with heparin which is introduced into it from a syringe armed with a fine injection needle that can readily be introduced into the free, pointed end of the cannula. It is important to aspirate into this syringe any air bubbles that may have entered into the bladder or the cannula in order

to prevent air embolisms. It is well to wipe off any excess heparin that may ooze out through the cannula, since a few drops of it suffice to prevent clotting, and an excess may lead to diffuse bleeding. Now follows the most dangerous part of the operation: The introduction of the pointed end of the cannula into the vena cava cranial from the place where the vessel was occluded and caudad from the loosely laid ligature underneath the ilio-lumbar vein. This is accomplished by firmly grasping the middle of the cannula with one forceps, and the vena cava with another, and then carefully pushing the point into the vessel until the circular groove underneath the pointed end is at the level of the loosely laid ligature. At this moment a few drops of blood usually escape from the vein, but bleeding is stopped immediately by pulling the ends of the loose ligature and thus fastening the wall of the cava to the cannula. A second knot fixes this ligature and thus a permanent anastomosis between urinary bladder and vena cava has been established. After removing the retractor and the cotton pads, which kept the intestines out of the way, the muscle and skin wounds are closed and the operation is finished. The intervention takes approximately fifteen minutes, and in the last series of fifty operations on rats, ranging between 105 and 275 Gm. of body weight, there was not a single case of death during the operation. Female animals are preferable because the uterus does not interfere with the cannula as much as the prostate and seminal vesicles, yet both sexes can be used.

After the animals recover from the anesthesia, they appear to be perfectly normal and readily take nourishment, showing no detectable signs of damage. Approximately twenty-four hours are required before the animals begin to show some tremor characteristic of uremia. The average survival time is thirty to forty-five hours.

Complete nephrectomy or ligature of the ureter is generally tolerated for a slightly longer time than the urinary-venous anastomosis. It is remarkable, however, that the continuous influx of urine into the blood is not accompanied by more rapidly developing signs of intoxication. It will be recalled that numerous investigators found urine to be rich in highly toxic substances, and workers who made a unilateral anastomosis between a ureter and a vein in the dog<sup>1-4</sup> concluded that introduction of the urine produced by one kidney suffices to elicit a rapidly developing fatal intoxication even if urine elimination through the other kidney proceeds normally. On the other hand, Geer and Dragstedt,<sup>5, 6</sup> who used a similar surgical procedure, observed no toxic symptoms and no rise in blood nonprotein nitrogen after unilateral anastomosis of a ureter and a vein in the dog. In certain cases their animals survived several weeks and were still in good condition at the time they were sacrificed. From this it would appear that urine in itself is not a highly toxic product.

The only investigator who attempted to connect both ureters with veins was de Almeida<sup>7, 8</sup> who, like all previous investigators, used the dog as an experimental animal. He employed a rather complicated method which involved the insertion of long tubes into the ureters, the tubes being led out of the peritoneal cavity and then introduced into the femoral vein. The procedure, which was successful only in a limited number of cases, led to the usual symptoms of

nremia and was fatal in a shorter period than complete nephrectomy. These experiments were also performed merely with the intent to study the toxicity of urine and, to our knowledge, the technique was never employed as a method for the selective differentiation between the excretory functions of the kidney on the one hand, and its endocrine and metabolic activities on the other hand.

The most striking pathologic changes, which we noted in our rats bearing a urinary-venous anastomosis, were a pronounced enlargement of the adrenal cortex, occasional lung edema, and a rise in the blood nonprotein nitrogen which was usually more pronounced than at a comparable period after complete nephrectomy. The marked decrease in blood hemoglobin, seen in the anastomosis-bearing animals, indicated, furthermore, that, owing to the continuous reflux of urine, blood dilution is more pronounced than after nephrectomy.

It is hoped that the use of this simple experimental technique, applicable even to small laboratory rodents, will prove useful in the study of the purely endocrine and metabolic functions of the kidney.

#### SUMMARY

A technique is described which permits the deviation of the entire urine into the inferior vena cava after ligation of the urethra by means of a cannula that connects the bladder with the vein. This technique permits the study of the endocrine and metabolic activities of renal tissue apart from its excretory function.

The expenses of this investigation were defrayed through a fund donated by the Desbergers-Blismol Co., Montreal, Canada.

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# THE USE OF A DETERGENT IN SOLUBILITY TESTS FOR THE IDENTIFICATION OF PNEUMOCOCCI\*

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THEORETICAL considerations suggested that synthetic surface-active agents might affect pneumococci in a manner similar to that of bile and its salts. It is well known that lysis of pneumococci is activated by any process that destroys viability without impairing antolytic enzyme-activity, while it is inhibited by conditions unfavorable to such activity.<sup>1-3</sup> Agents that will induce the dissolution of pneumococci are legion. The principal ones, as listed by White,<sup>4</sup> are bile acids and salts, dilute sodium hydroxide, unsaturated fatty acids, soaps, and saponin. The most satisfactory among these is the bile salt, sodium desoxycholate, which brings about the dissolution of pneumococci at room temperature in a very few minutes.

Most of the agents mentioned depress the surface tension of the solution in which they are dissolved. The recently developed surface-active agents, some of which are long-chain alcohol sulfates, are strong surface-tension depressants. Cowles<sup>5</sup> showed that "duponols," in a concentration of only 0.03 per cent, prevent the growth of gram-positive bacteria. One such compound, duponol WA (flakes),† was employed by us in solubility tests.

## EXPERIMENTAL WORK

A 0.2 per cent solution of duponol in distilled water was found to have exactly the same activity as a 1 per cent solution of sodium desoxycholate. When higher and lower concentrations were employed, the clearing of the solution was slower. A 0.2 per cent solution is opalescent, but when it is added to broth cultures, this property disappears. Sterilization before use is unnecessary. The solution has not been found to deteriorate, even if stored for several months. The microorganisms were cultivated in an infusion broth that contained 2 per cent peptone, but no serum, and only 0.02 per cent added dextrose. The addition of dextrose to the medium may be omitted. The dissolution of pneumococci in eighteen-hour-broth cultures was observed grossly and also by examination of films stained by Gram's method and prepared immediately after the addition of 0.1 ml. of duponol or sodium desoxycholate to 0.6 ml. of culture. Films were also prepared at the end of five minutes and of one hour.

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†A detergent manufactured by DuPont.

Twelve strains of pneumococci, comprising seven different types, dissolved completely in less than five minutes at room temperature under the influence of either duponol or sodium desoxycholate. All were insoluble when previously heated to 75° C. for twenty minutes. One type-specific type 14 strain and two degraded strains that had lost type specificity dissolved incompletely in both reagents, while two other strains, supposedly representing completely degraded pneumococci, appeared to be insoluble in both. Eleven strains of streptococci producing greenish discoloration of blood agar, and one strain each of hemolytic streptococcus, nonhemolytic streptococcus, and Friedländer's bacillus were insoluble in both reagents.

The pH of an eighteen-hour broth culture of pneumococcus is about 7.0 or 7.2. It is not influenced appreciably by adding the bile salt. It is lowered slightly when duponol is added, but remains well within the range of optimal enzymatic activity. The surface tension of the broth used was found to be 47.1 dynes per centimeter. The addition of sodium desoxycholate lowers the tension to approximately 44; and of duponol, to approximately 36.

Duponol was used in parallel tests with sodium desoxycholate during a four-month period in the routine examination of specimens received for pneumococcus typing. Its activity was found to compare very favorably with that of the bile salt. Broth cultures of fishings of colonies surrounded by a zone of greenish discoloration were tested; eighty-five individual strains were soluble in both reagents, two were partially soluble in both, and 78 were insoluble in both. In no instance was a discrepancy observed.

The low cost of duponol WA (flakes) compared with that of sodium desoxycholate is its principal advantage. The method has now been adopted as an alternative standard procedure in solubility tests for pneumococci in this laboratory.

#### SUMMARY

A detergent, duponol WA (flakes), in 0.2 per cent aqueous solution, has been found to have the same activity as 1.0 per cent sodium desoxycholate in solubility tests for differentiating pneumococci from other microorganisms producing greenish discoloration on blood agar. It has the advantage of being very inexpensive.

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## CHEMICAL

### THE NORMAL VALUE FOR THE HIPPURIC ACID LIVER FUNCTION TEST\*

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QUICK<sup>1</sup> first described the hippuric acid liver function test in 1933. He stated that under the conditions of the test the normal excretion of benzoic acid as hippuric acid is 3 to 3.5 Gm. In 1936 he<sup>2</sup> suggested that the efficiency of the liver be expressed as a percentage, using 3 Gm. of excreted benzoic acid as normal. On this basis he gave the normal range as 85 to 110 per cent. In 1940 he<sup>3</sup> improved the original method by the addition of 5 Gm. of ammonium sulfate for each 10 c.c. of urine to decrease the solubility of the hippuric acid. At this time he gave 90 to 120 per cent as normal excretion, and suggested that an excessive excretion, i.e., over 120 per cent, might be abnormal.

After we started using the ammonium sulfate method routinely, we noticed that many of our values were over 120 per cent. Some were as high as 150 per cent. Several patients who had previously been tested by the old method gave consistently higher results with the new method. In some cases the variation between the two methods amounted to 20 per cent. Theoretically, the difference between the additive corrective factors (0.33 Gm. in the old method and 0.1 Gm. in the new), used for the amount of hippuric acid remaining in solution in the urine, should make the results by the two methods essentially identical. Since this was not found to be the case, it seemed desirable to investigate the normal excretion of hippuric acid and the factors that may influence it.

#### PROCEDURE

Hippuric acid tests were run on 30 men and 25 women, all presumably normal, and between the ages of 20 and 30 years, the majority being between 20 and 25 years of age. The subjects were either students or laboratory technicians. Six grams of sodium benzoate were dissolved in 30 c.c. of water and taken after breakfast. This was followed by one-half glass of water. The bladder was emptied immediately before the test, and hourly urine specimens were collected for four hours. Each specimen was measured and, if over 100 c.c., it was acidified with a few drops of acetic acid and concentrated over a water bath to about 50 c.c. The four specimens were pooled, as recommended

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by Snell and Plunkett.<sup>4</sup> The pooled specimen was measured and divided in equal parts. To one portion 5 Gm. of solid ammonium sulfate were added for each 10 c.c. of urine. When the salt was dissolved, the urine was filtered and then acidified with concentrated hydrochloric acid until the urine was distinctly red to Congo red paper. After vigorous stirring, the specimen was allowed to stand for thirty minutes in the icebox before separating the crystalline hippuric acid by filtration through a Buchner filter. The crystals were washed with ice cold water, then dissolved in hot water. The amount of hippuric acid was determined by titrating with 0.2 N. sodium hydroxide, using phenolphthalein as an indicator. One cubic centimeter of 0.2 N. sodium hydroxide equals 0.0358 Gm. of hippuric acid. This method checked very well with the gravimetric method. We had adopted the titration method, since most clinicians in hospitals like their reports the same day the test is performed; and with the gravimetric method it was necessary to let the hippuric acid dry overnight in order to obtain uniform results. The aliquot portion was treated in a similar manner except that no ammonium sulfate was added. The calculation for the two methods is identical except for the addition of the amount of hippuric acid remaining in solution in the urine. With the method using ammonium sulfate, 0.1 Gm. of hippuric acid is added for each 100 c.c. of urine, and 0.33 Gm. per 100 c.c. is added in the method not using ammonium sulfate. These are the amounts assumed by Quick to remain in solution. The amount of hippuric acid was multiplied by 0.68 to change it to benzoic acid.

#### DISCUSSION

Not all the hippuric acid excreted in the urine is precipitated by the methods used. Quick, therefore, corrected his results by adding 0.33 Gm. per 100 c.c. of urine to the value found without the addition of ammonium sulfate, and 0.1 Gm. to the value obtained when the sulfate was added. The results with each method are stated as a percentage, obtained by dividing the "corrected" amount of benzoic acid excreted as hippuric acid by the arbitrarily chosen normal excretion of 3 Gm., and multiplying by 100.

The estimated amount of benzoic acid excreted as hippuric acid in the group of women ranged from 75 to 149 per cent with the old method, that is, the method not using ammonium sulfate. In the group of men the amount ranged from 110 to 156 per cent. With the new method, using ammonium sulfate, the range of the estimated amount for women was 95 to 155 per cent and, for men, 114 to 166 per cent. The difference between the two methods for each person varied greatly. The greatest increase of the ammonium sulfate method over the old method was 33 per cent. In four cases results by the old method were greater than those obtained by the use of ammonium sulfate. However, the uncorrected value in each case was greater by the latter method, the difference being due to the larger quantity (0.33 Gm.) added to "correct" the value by the method not using ammonium sulfate. The variation in the two methods can be explained on the basis of the variability in the precipitation of the hippuric acid in the urines without the addition of ammonium sulfate. The addition of ammonium sulfate to the urine gives a more con-

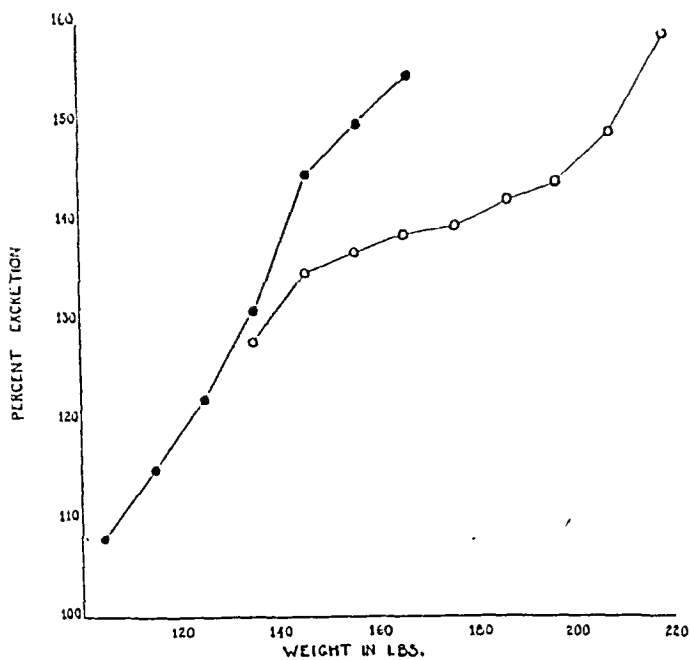


Fig. 1.—Per cent of excretion of hippuric acid as benzoic acid in relation to weight.

•—• Women.  
○—○ Men.

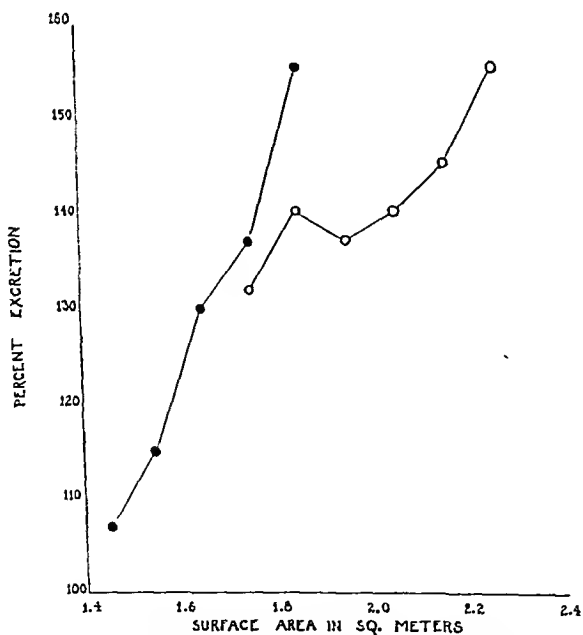


Fig. 2.—Per cent of hippuric acid as benzoic acid in relation to surface area.

•—• Women.  
○—○ Men.

sistent and accurate estimation of the hippuric acid. Weichselbaum and Probst<sup>5</sup> demonstrated that, with Quick's original method, the amount of hippuric acid that remained in solution was greater than 0.33 Gm. per 100 c.c. and showed a large range of variability with different urine specimens. They suggested using sodium chloride to decrease the solubility of hippuric acid. However, Marron<sup>6</sup> found that ammonium sulfate was the best salt to use for reducing the solubility of hippuric acid.

The statistical analysis of the data obtained in these tests is shown in Table I. From this table it is seen that the mean body weight, the mean surface area, and the mean per cent excretion is much greater in men than in women. The coefficients of correlation between body weight and per cent excretion (+0.873) and between surface area and per cent excretion (+0.834) in the women of this group was much higher than in the men (+0.545 and +0.429, respectively). The coefficient of correlation between body weight and per cent excretion in the men, in the women, and in the combined group was higher than that between surface area and per cent excretion in the same groups.

TABLE I  
STATISTICAL ANALYSIS OF DATA

|                                      | MEN            | WOMEN          | MEN AND WOMEN  |
|--------------------------------------|----------------|----------------|----------------|
| Body weight and per cent excretion*  |                |                |                |
| Body weight                          |                |                |                |
| Mean                                 | 172.60 ± 2.57  | 125.80 ± 2.12  | 152.60 ± 2.71  |
| Standard deviation                   | 21.00 ± 1.84   | 16.48 ± 1.57   | 29.74 ± 1.91   |
| Per cent excretion                   |                |                |                |
| Mean                                 | 140.86 ± 1.60  | 124.60 ± 2.04  | 137.60 ± 1.47  |
| Standard deviation                   | 12.44 ± 1.08   | 15.86 ± 1.51   | 16.10 ± 1.09   |
| Correlation coefficient              | +0.545 ± 0.095 | +0.873 ± 0.032 | +0.677 ± 0.049 |
| Surface area and per cent excretion* |                |                |                |
| Surface area                         |                |                |                |
| Mean                                 | 1.99 ± 0.02    | 1.62 ± 0.016   | 1.892 ± 0.022  |
| Standard deviation                   | 0.147 ± 0.02   | 0.12 ± 0.011   | 0.229 ± 0.015  |
| Per cent excretion                   |                |                |                |
| Mean                                 | 140.86 ± 1.60  | 124.80 ± 2.12  | 137.60 ± 1.47  |
| Standard deviation                   | 12.44 ± 1.08   | 16.48 ± 1.57   | 16.10 ± 1.09   |
| Correlation coefficient              | +0.429 ± 0.110 | +0.834 ± 0.041 | +0.613 ± 0.058 |

\*Per cent excretion =  $\frac{\text{Number of grams excreted as benzoic acid}}{3 \text{ Gm.}} \times 100.$

The relation of excretion to body weight is also shown in Fig. 1. These curves were made from weighted averages for each 10 pounds of weight for the men and women separately. None of these persons were obese. The variations in weight were due to differences in general build, muscular development, and stature. In such a group the size of the organs may be assumed to vary directly with the size of the body as a whole. A person with a large well-developed body will have a larger liver. This being true, it may be assumed that the liver forms hippuric acid from benzoic acid in proportion to its size. It is not surprising, therefore, to find that the per cent of benzoic acid excreted as hippuric acid is greater in larger built persons than in smaller built ones. For this reason, the normal standard for such excretion should not be fixed within even fairly wide limits but should be related to the body weight of the patient.

It is interesting to note that the coefficient of correlation between per cent of excretion and both the body weight and the surface area was much higher in the women of this group than in the men. This is also shown in Figs. 1 and 2. The curve for the women is much steeper and straighter in each case than that for the men. This may be due in part to wider range of weights of the men (135 to 220, or 85 pounds) as compared with the range in the women (103 to 167, or 64 pounds). However, while the actual surface areas of the men were, in general, greater than in women, the spread between the extremes in each sex was almost identical—for women, 1.43 to 1.87, or 0.44 sq. m.; for men, 1.78 to 2.26, or 0.48 sq. m.

We do not agree with Rosenberg and Soskin<sup>7</sup> that a hypersecretion of hippuric acid is pathologic, at least in all cases. We believe that the weight of the patient must be taken into consideration. Although Quick<sup>5</sup> definitely stated in one of his early articles that the quantitative results indicated that the excretion of hippuric acid depended on the size of the person, that is, on the surface area, this has not been emphasized in the routine use of the test. Consequently high values have been misinterpreted.

#### CONCLUSIONS

1. The addition of ammonium sulfate to the urine to decrease the solubility of hippuric acid makes the hippuric acid liver function test more accurate and consistent in results.

2. The lower limit of normal when ammonium sulfate is used in the test should be 90 per cent.

3. There should be no upper limit for normal because there is a correlation between body weight and the benzoic acid excreted as hippuric acid.

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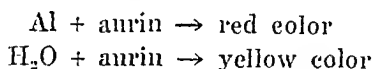
# DETERMINATION OF ALUMINUM IN BIOLOGICAL MATERIAL WITH THE EVELYN PHOTOELECTRIC COLORIMETER\*

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ALTHOUGH numerous quantitative tests for aluminum have been described in the literature, no method has proved entirely satisfactory. A major technical problem has been the development of a stable, colored lake free of interfering substances and suitable for colorimetric analysis. In the course of a study on the effect of aluminum hydroxide and aluminum phosphate on mineral excretion in man, an improved colorimetric technique for the determination of aluminum was developed which overcomes this difficulty and permits the rapid and accurate quantitative measurement of aluminum in biological material.

## PRINCIPLE OF PROCEDURE

The material to be tested for aluminum is put into solution and brought to a pH of 4.9. Aurintricarboxylic acid is added, and the mixture is heated to from 70° to 78° C. until a red color develops, the intensity of which is measured in a colorimeter.



Rapid and reproducible reading of samples for their aluminum content is further facilitated by a chart which converts the values obtained from the colorimeter into milligrams of aluminum. This chart may be prepared by plotting the readings of light intensity obtained from solutions of known aluminum content on semilogarithmic paper, and interpolating from the standard curve.

## DEVELOPMENT OF A STANDARD CURVE

### Reagents:

1. *N/10 HCl*—8.3 ml. of 37 per cent (concentrated) HCl (reagent grade) diluted to one liter with distilled water.
2. *Aluminum standard*—3.5018 Gm. of  $\text{K}_2\text{SO}_4 \cdot \text{Al}_2(\text{SO}_4)_3 \cdot 24\text{H}_2\text{O}$  are dissolved and diluted to one liter with *N/10 HCl* (stock solution). Dilute 1:20 with *N/10 HCl* to give a solution containing 0.01 mg. Al per milliliter.
3. *Phthalate buffer*—*M/5* potassium acid phthalate ( $\text{KHC}_8\text{H}_4\text{O}_4$ ). 20 Gm. of potassium biphthalate in 500 ml. of water.  
250 ml. of *M/5* phthalate and 238 ml. of *N/10 NaOH* made up to one liter with water. The pH should be 4.9 to 5.1.

\*From the Frank Billings Medical Clinic, Department of Medicine, University of Chicago. This work was supported by a grant from John Wyeth & Brother, Inc., Philadelphia, Pa. Received for publication, May 18, 1942.

4. *0.1 per cent aurintricarboxylic acid*—(Eastman Kodak P 4468.)  
1 Gm. of aurin and 2.5 ml. of concentrated  $\text{NH}_4\text{OH}$  diluted to one liter with water. Keep in a brown bottle. A good quality of aurin gives a straw-yellow color in a dilution of 1:100 ml.
5. *1:50  $\text{NH}_4\text{OH}$* —1 ml. of concentrated (28 per cent)  $\text{NH}_4\text{OH}$  made up to 50 ml. with water.
6. *Methyl red*—0.2 per cent alcoholic solution.
7. *Ammonium thiocyanate* (or potassium thiocyanate)—60 per cent. Keep in a brown bottle.
8. *Ether*—exposed to light or containing some alcohol.

#### Standards:

Ten standards of varying concentrations of aluminum are prepared from the stock solution to contain from 0.005 mg. per milliliter to 0.05 mg. per milliliter.

#### Blank:

Since the pH affects the color of the aurin itself without the addition of aluminum, it is important to have the blank at the same pH as the standards or unknowns. Accordingly, the blank contains all the reagents except the aluminum.

#### PREPARATION OF A STANDARD CALIBRATION CURVE

0.42 ml. of 1:50  $\text{NH}_4\text{OH}$ \*  
5.00 ml. of phthalate buffer  
1.0 ml. of standard  
1.0 ml. of aurin

Three such tubes are prepared for each dilution of standard in addition to three blanks. The blank contains 1 ml. of tenth-normal hydrochloric acid in place of a standard solution. After the addition of the aurin the tubes are shaken gently to mix the contents and placed in the test tube rack. When all tubes are set up and stoppered, the rack is placed in a water bath for exactly five minutes at a temperature between  $70^\circ$  and  $78^\circ \text{C}$ . The rack with the tubes is then removed from the bath and immersed in a deep pan of cold water for five minutes, or until the tubes reach room temperature. The water level in the pan should extend at least to the level of the solution inside the tubes. The solution is then diluted to the final volume of 25 ml. The first few milliliters are used to wash down any solution condensed on the walls of the tube into the main body of the liquid, and then the remainder is allowed to run freely into the middle of the tube to aid in mixing the solution. The tube is then moved in a circular motion until the color is evenly distributed throughout. Inverting the tube to mix the contents is faster, but any loss of fluid introduces an error in the reading. The standards are then ready to be read in the colorimeter. The reading of the "blank" is subtracted from that of each standard. The galvanometer readings, indicating the amount of light transmitted, are then plotted against the concentration of aluminum, using semilogarithmic paper. The values from this prepared chart can be used indefinitely to determine the concentration of aluminum in any unknown solution prepared under identical conditions.

\*One milliliter of standard requires 0.42 ml. of 1:50  $\text{NH}_4\text{OH}$  for neutralization to methyl red.

## CENTER POINT

The colorimeter is adjusted to read 100 per cent light transmission with the tube containing water in the path of the light. The tube is then removed and the galvanometer reading taken as the "center point." The same "center point" is kept throughout the reading of all the unknowns prepared at the same time.

TABLE I

RECOVERY OF ALUMINUM IN THE FECES DURING ORAL ADMINISTRATION OF ALUMINUM HYDROXIDE AND ALUMINUM PHOSPHATE

| ANTACID GIVEN      | CASE I                   |                          | CASE II                  |                          |
|--------------------|--------------------------|--------------------------|--------------------------|--------------------------|
|                    | 4-DAY INTAKE<br>Al (MG.) | 4-DAY OUTPUT<br>Al (MG.) | 4-DAY INTAKE<br>Al (MG.) | 4-DAY OUTPUT<br>Al (MG.) |
| Aluminum hydroxide | 4,716                    | 4,866                    | 4,716                    | 4,986                    |
| Aluminum phosphate | 2,625                    | 2,787                    | 2,625                    | 2,697                    |
| Control            |                          | 13                       |                          | 15                       |

TABLE II

RECOVERY OF ALUMINUM CHLORIDE ADDED TO SAMPLES OF FECES

|                | ALUMINUM ADDED<br>(MG./ML.) | ALUMINUM FOUND<br>(MG./ML.) |
|----------------|-----------------------------|-----------------------------|
| 2 Gm. of feces | 0.0120                      | 0.0123                      |
| 2 Gm. of feces | 0.0120                      | 0.0122                      |
| 2 Gm. of feces | 0.0120                      | 0.0122                      |

Blank determinations on diluted urines, plasma, feces and distilled water, known to be free of aluminum but ashed, extracted, and treated with the reagents, gave values of 0.0003 to 0.0003 mg. per milliliter.

## PREPARATION AND ANALYSIS OF UNKNOWN

*Feces.*—The quantity of feces used for analysis depends upon the conditions of the clinical experiment. A patient on an ordinary diet excretes a very small amount of aluminum in the stool, so that it is advisable to use 10 Gm. (wet weight) of feces for an analysis. During aluminum therapy 2 Gm. of feces are sufficient.

For *ashing*, 2 Gm. of fresh, moist, well-mixed feces are weighed into a vitreosil silica beaker and 0.2 ml. of nitric acid, and 0.2 ml. of sulfuric acid are added. The water is driven off by heating at 100° C. in a drying oven. It is then ashed in an electric muffle furnace for about twenty-four hours at a temperature of 400° to 500° C. The heat should be started slowly to avoid spattering of the material. When a fluffy white ash is obtained, 10 ml. of tenth-normal hydrochloric acid are added to the ash, which is transferred to a centrifuge tube where the insoluble material is separated by centrifuging. According to the expected concentration of aluminum, aliquots of the supernatant fluid are further diluted 25 to 50 times.

If iron is present in the solution, it can be removed after bringing it to volume by adding 60 per cent ammonium thiocyanate and ether to a portion and shaking it several times in a separatory funnel or centrifuge tube. The colored ether extract is siphoned off, and the aluminum is determined on 1 ml. aliquots of the colorless substrate, exactly as in the calibration of the standard curve.



*Urine.*—One hundred grams of urine are weighed into a vitreosil beaker, 0.2 ml. of concentrated sulfuric acid and 0.2 ml. of nitric acid are added, and the solution is evaporated to dryness, charred in a drying oven, and then ashed in a muffle furnace for about twenty-four hours at a temperature between 400° and 500° C. Five milliliters of tenth-normal hydrochloric acid are added to the ash. The solution is then centrifuged. Two milliliters duplicate samples of the clear supernatant liquid are placed in colorimeter tubes and evaporated to dryness in an oven at 100° C. One milliliter of tenth-normal hydrochloric acid is then added to the dried residue in the tubes, and the color is developed exactly as for feces. It is sometimes advisable to combine the ash of several samples to increase the concentration of aluminum. Another method is to add a known amount of aluminum to bring the color into a better range for reading.

The concentration of aluminum in the urine is extremely low (0.005 to 0.15 milligrams per liter) even during aluminum therapy, and there are large variations from day to day in the same person on a constant intake of aluminum.

TABLE III

RECOVERY OF ALUMINUM IN URINE DURING ORAL ADMINISTRATION OF ALUMINUM HYDROXIDE AND ALUMINUM PHOSPHATE

| ANTACID GIVEN      | CASE I                        |  | CASE II                       |  |
|--------------------|-------------------------------|--|-------------------------------|--|
|                    | 24-HOUR<br>INTAKE<br>Al (MG.) | 24-HOUR OUT-<br>PUT IN URINE<br>Al (MG.) | 24-HOUR<br>INTAKE<br>Al (MG.) | 24-HOUR OUT-<br>PUT IN URINE<br>Al (MG.) |
| Control            | --                            | 0.014<br>0.015                           | --                            | 0.052<br>0.023                           |
| Aluminum hydroxide | 1,179<br>1,179                | 0.043<br>0.125                           | 1,179<br>1,179                | 0.075<br>0.091                           |
| Aluminum phosphate | 656<br>656                    | 0.041<br>0.222                           | 656<br>656                    | 0.125<br>0.139                           |

TABLE IV

DETERMINATIONS OF ALUMINUM IN THE BLOOD OF PATIENTS ON ANTACID THERAPY

| CASE | CONTROL<br>Al (MG./100 ML.) | TYPE OF THERAPY                        |  |
|------|-----------------------------|--|--|
|      |                             | ALUMINUM PHOSPHATE<br>(MG./100 ML. Al) | ALUMINUM HYDROXIDE<br>(MG./100 ML. Al) |
| I    | 0.020                       | 0.040                                  | 0.045                                  |
|      | 0.015                       | 0.045                                  | 0.050                                  |
|      | 0.022                       | 0.049                                  | 0.048                                  |
| II   | 0.050                       | 0.053                                  | 0.070                                  |
|      | 0.045                       | 0.046                                  | 0.049                                  |
|      | 0.048                       | 0.049                                  | 0.050                                  |
| III  | 0.030                       | 0.040                                  | 0.045                                  |
|      | 0.022                       | 0.030                                  | 0.040                                  |
|      | 0.040                       | 0.046                                  | 0.048                                  |

*Blood.*—Fresh oxalated blood is mixed well and centrifuged. Ten to 15 ml. of plasma are placed in a vitreosil silica beaker, 0.2 ml. of nitric acid and 0.2 ml. of sulfuric acid are added, and the solution is dried in an oven at 100° C. The sample is then ashed in a muffle furnace for twenty-four hours at 400° C., or until the ash is white. The ash is dissolved in 5 ml. of tenth-normal hydrochloric acid and transferred to a centrifuge tube. It is then centrifuged to remove the insoluble material. Two milliliter duplicates of the clear super-

nantant liquid are placed in colorimeter tubes and evaporated to dryness in an oven at 100° C. When dry, 1 ml. of tenth-normal hydrochloric acid is added, and the other reagents, as for the urine and feces.

Amounts of aluminum recovered from the blood of patients excreting about 2 to 4 mg. per twenty-four hours in the feces vary from 0.0113 to 0.0984, with an average of 0.045 mg. per 100 ml.

*Recovery Experiment.*—A solution containing a known amount of aluminum was added to samples of plasma and ashed, as described above. The results are recorded in Table V.

TABLE V  
RECOVERY OF ALUMINUM ADDED TO PLASMA

| ALUMINUM<br>IN PLASMA | ALUMINUM<br>ADDED (MG.) | ALUMINUM<br>RECOVERED (MG.) | ALUMINUM<br>CALCULATED (MG.) |
|-----------------------|-------------------------|-----------------------------|------------------------------|
| 0.0180                | 0.0050                  | 0.0230                      | 0.0230                       |
| 0.0175                | 0.0050                  | 0.0224                      | 0.0225                       |
| 0.0510                | 0.0050                  | 0.0554                      | 0.0560                       |
| 0.0540                | 0.0050                  | 0.0591                      | 0.0590                       |

#### EXPERIMENTAL CONSIDERATIONS

1. *Influence of pH and temperature on the color of the lake formed by the aluminum-aurin complex.* Eveleth and Myers concluded,<sup>3</sup> after a critical review of the colorimetric analyses for aluminum currently in use, that the lake formed with aurintricarboxylic acid as a chromatogenic agent was superior to that formed with alizarin, or with 1, 2, 5, 8-hydroxyanthraquinone. On the basis of their work, aurin was used in this test. It is not an ideal reagent for colorimetric work, however, since it is not colorless and varies with the pH and temperature. Thus, in a solution of pH 7.0 or above, it is yellow; at a pH of 3 to 5 it has a faint orange-pink color. Various investigators have sought to overcome this difficulty by suggesting a final adjustment of the solution to a pH 7.2, using ammonium chloride for the purpose of removing any excess color. This procedure is difficult to control, since the lake is readily decomposed or its color progressively fades.

In the present test the pH of the aluminum solution was adjusted before the color was developed and maintained at a constant hydrogen-ion concentration by means of a buffer. The acid solution of digested biological material was made neutral to methyl red with a small amount of ammonium hydroxide. A phthalate buffer at pH 4.9 to 5.1 was used because potassium biphthalate was easier to handle than the hygroscopic ammonium acetate used by some investigators for an acetate buffer. By the foregoing procedure a solution was obtained which had a final pH of 5.1. At this pH a definite red lake formed in the presence of aluminum. The color, however, developed slowly at room temperature. When heated in a water bath at 70° to 78° C., it developed a maximum intensity in five minutes.

2. *Prevention of the precipitation of the colloidal color complex at high concentrations of aluminum.* Previous investigators reported precipitation of the lake when the concentration of aluminum exceeded 0.015 mg.<sup>22</sup> To obtain a curve for the photoelectric colorimeter, it was desirable to keep the lake in a

dispersed phase well beyond a concentration of 0.05 mg. The stability of the lake was not increased by changes in pH after the development of color; it was less stable when large amounts of ammonium salts were added. Attempts to peptize the precipitate by adding large amounts of water were unsuccessful. Using organic solvents in diluting the colored lake did not prevent it from precipitating. Heating above 80° C. increased the tendency of the lake to precipitate. When more than 1 ml. of aurin was used, it was less stable.

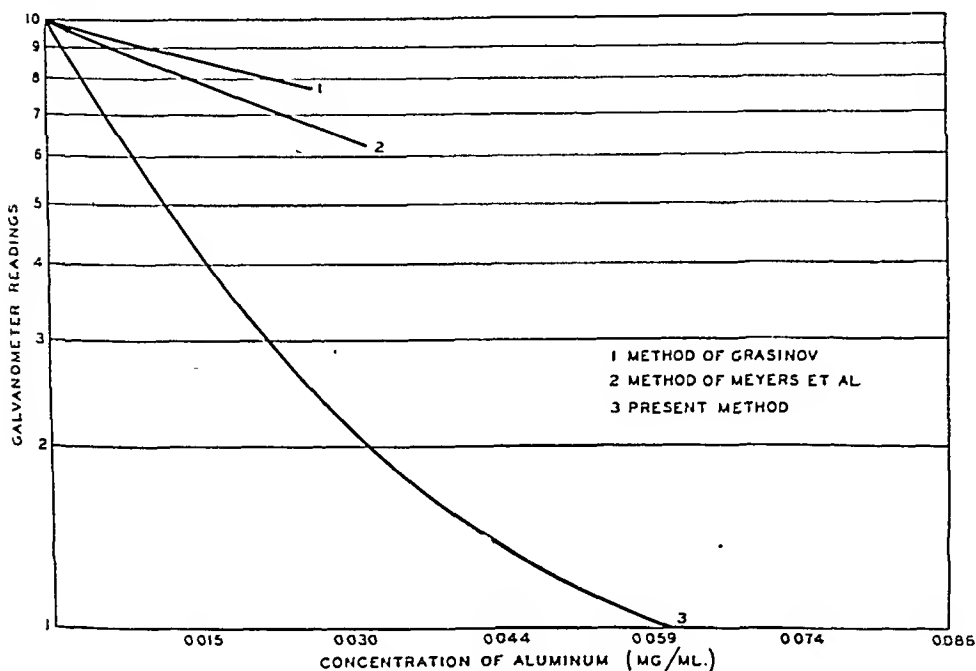


Chart 1.—Comparison of methods for sensitivity.

This problem was solved by the following procedure: The aluminum-aurin lake was allowed to form in a volume of solution not exceeding 10 ml., and was heated at 70° to 78° C. for five minutes. After cooling, it could be diluted with water to any desired volume without danger of precipitation. A solution containing as much as 0.063 mg. of aluminum in 25 ml. remained stable for an indefinite period of time (Chart 1).

3. *Optimum quantity of aurin reagent for developing a color with aluminum.* Amounts of aurin reagent, varying from 0.16 ml. to 2.0 ml., were tested. One milliliter yielded the best results. An amount of aurin less than 1 ml. was insufficient to contrast differences in concentration of aluminum; more than 1 ml. gave too much color to the blank.

4. *Elimination of interfering substances.* Yoe,<sup>23</sup> after investigating certain metallic ions which might form colored lakes similar to that of aluminum with aurin, found that all except iron were decolorized by ammonia.

A qualitative test to determine whether iron is present in the sample should be made before the development of color. Sixty per cent of ammonium thiocyanate, followed by an ether extraction, completely removed the interfering substance when present.

5. *The final dilution.* For the greatest accuracy in photoelectric colorimetry, the intensity of light transmitted by the colored solution should cause a deflection of the galvanometer needle within the most sensitive range of the instrument. Most of the galvanometer readings should fall, therefore, between 80 and 20 on the galvanometer scale. The final volume of 25 ml. was found, experimentally, to meet this requirement.

6. *Selection of filter.* The filter was selected from a spectrophotometric analysis of the colored solution. The characteristic peak of maximum transmission was between 520 and 540  $m\mu$ , at about 530  $m\mu$ . The Evelyn filter No. 520 was slightly more sensitive than No. 540.

#### COMMENT

Analyses of feces, urine, and plasma for aluminum before and after the administration of aluminum phosphate and aluminum hydroxide indicate that the aluminum is excreted entirely by way of the gut and that there is very little, if any, absorption into the blood stream. Kehoe and co-workers<sup>7</sup> found "the daily output in the feces . . . practically equivalent to daily intake in the diet," when speaking of the trace metals, such as aluminum, normally found in the body. In agreement with this observation the figures in Table I show a quantitative recovery of aluminum in the feces during aluminum therapy.

These same authors<sup>7</sup> report the concentration of aluminum in normal urine as 0.052 mg. per liter  $\pm$  0.025 mg. The values given for control urines in Table III of the present study are 0.015 mg. and 0.052 mg. Since the concentration of aluminum in the urine varies from day to day in the same person on a constant diet, sharp margins of normal are not easily established.

Grasinov<sup>4</sup> reported 0.068 to 0.0708 mg. of aluminum in 100 Gm. of whole blood. Kehoe<sup>7</sup> found 0.015 mg.  $\pm$  0.012 mg. of aluminum per 100 Gm. of whole blood "occurring almost entirely in the plasma." The values in Table IV of this present study, based on analyses of plasma, range from 0.015 to 0.050 mg. per 100 c.c.

From these comparisons it may be concluded that the results of the present method are in agreement with those of previous investigators.

#### CONCLUSIONS

1. An improved method for the quantitative determination of aluminum in biological material, using the Evelyn photoelectric colorimeter, is described. The use of a phthalate buffer, a smaller volume of reaction mixture, and careful control of the pH and temperature of the solution, as well as of the time of dilution, contribute to the formation of a stable-colored solution. The procedure is easily performed and yields accurate and reproducible results.

2. There is no significant increase in the aluminum content of the blood or urine after the oral administration of aluminum hydroxide or aluminum phosphate.

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# MEDICAL ILLUSTRATION

## THE BASIC TECHNIQUE OF KODACHROME PHOTOGRAPHY OF FLUORESCENCE PHENOMENA\*

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UNTIL recently the photography of fluorescence phenomena was seriously handicapped since it was unable to reproduce accurately a variety of colors. It was possible to record some of these color differences by means of black-and-white film.<sup>1, 3, 4</sup> However, this method is truly colorless and falls flat when compared with the spectacular and delicate shades of color that have been observed and described as a result of fluorescence in near-ultraviolet radiations. The relatively recent development of practical, efficient sources of near-ultraviolet radiations has given a great impetus to fluorescence studies in many fields, and especially in the field of medical examinations and diagnosis.<sup>1, 2, 4, 5</sup> An accurate method of reproducing colors is the result of the development of Kodachrome film. These two developments of suitable lighting and film have gone hand in hand and each serves to amplify the utility of the other.

The simple efficient apparatus and filters necessary to extend the limited range of visual perception by means of fluorescence phenomena has been reported previously.<sup>2</sup> It is the purpose of this paper to describe and discuss equally simple methods and apparatus for accurately and faithfully recording fluorescence phenomena by means of Kodachrome photography.

Fluorescence is the term applied to the phenomenon which certain substances or bodies show when illuminated with ultraviolet light. It usually involves the absorption of the invisible near-ultraviolet radiations and the emission of visible light of longer wave length. For a more complete discussion, the reader is referred to other literature on this subject.<sup>1, 2, 4</sup>

One of the difficulties that had to be overcome in the photography of fluorescence was the fact that the ordinary film or plate is less sensitive to the rays of visible light caused by fluorescence than it is to the reflected rays of the near-ultraviolet or so-called actinic rays used to excite the fluorescence. The problem was to find a filter to place either in front or in back of the lens of the camera to absorb the reflected near-ultraviolet radiations and to transmit fully the relatively feeble rays of visible light caused by fluorescence. The photography of fluorescence must be done in a dark room. No extraneous or stray light can

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†One of us (F. H. J. Figge) is indebted to Dr. C. N. H. Long and the staff of the Physiological Chemistry Department at Yale University School of Medicine, who first demonstrated the feasibility of Kodachrome photographs of porphyrin incrustations in rats.

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be tolerated because of the long exposures necessitated by the low intensity of the light emitted by fluorescent objects. The light source must be intense, especially in the near-ultraviolet range (3,000 to 4,000 A.U.). The primary filter should exclude visible light from the fluorescent object. It should also exclude the far-ultraviolet or tanning and bactericidal rays. This is desirable from the point of view of safety for the photographer and others who may be present.

The photography of fluorescence thus requires, in addition to an ordinary camera, a lamp radiating only ultraviolet or invisible radiations, and a secondary filter which transmits visible light. The desirable characteristics of each of these requirements will be discussed in the course of a description of the apparatus and technique used to take the Kodachrome fluorescence photographs shown in Plate I.

The light source for Kodachrome fluorescent photography may be any type of quartz mercury arc or even carbon arc. Both of these are strong in the near-ultraviolet wave lengths. The primary filter must be applied in such a way as to prevent the leakage of visible light. For this reason the mercury arc is more convenient to use. There are numerous lights on the market today that are designed particularly for fluorescence studies. These have filters built into the lamp, either as close fitting lenses or as an envelope which almost completely surrounds the quartz mercury arc. It is, therefore, advisable to discuss the light source along with the primary filter.

These lamps, with the attached filter, which are designed for fluorescence studies are decidedly superior to many others for photographic utility.\* Two of these lamps were used to irradiate the object with near-ultraviolet light in order to obtain the fluorescence photograph in Plate I. One was fixed at a distance of 20 inches from the object, and the other was held in the hand at the same distance and moved from side to side to avoid deep shadows. The bulb of the lamp used consisted of a quartz mercury arc surrounded by a primary filter or envelope of filter glass. This was screwed into a socket on an alzac aluminum reflector. One of the excellent features of this lamp is that it may be moved from side to side or fastened to a ring stand to illuminate the object from any desired angle. It is absolutely necessary to irradiate all sides of an object to be photographed. Due to the mobility of this lamp only one is required, but in order to decrease the exposure time two lamps are advantageous.

The only undesirable feature is that the arc light is enclosed with filter glass similar to No. 587 Corning filter, and this filter is not interchangeable. It transmits a small amount of violet, green, and a trace of red light. Even this almost negligible amount of visible light interferes with the recording of purely fluorescent light. When the light from this lamp was examined with a diffraction grating spectroscope, the violet band at 4,100 A.U. was easily discernible. The red was dim and the green line of the mercury spectrum was only observed when the arc was in line with the slit on the spectroscope. In this connection the Corning filter No. 586 might be more desirable as a primary filter. This is more nearly monochromatic since it transmits no visible light. The near-ultraviolet transmission is, however, much lower than in glasses which transmit traces of visible light. Other Corning filters which are usually recom-

\*Detailed specifications will be furnished on request.

mended for fluorescence studies are the red-purple ultra No. 597, red ultra No. 584, blue-purple ultra No. 585, and red-purple corex A No. 986. While these filters are suitable for some studies in fluorescence, they are not suitable for color photography of fluorescence because of the visible light which they transmit.

The secondary filter used to photograph the fluorescence (Plate I) was a Corning glass filter No. 306 Noviol shade O. This has been referred to as the lens filter because it is placed either in front or in back of the lens. The perfect camera lens filter or secondary filter for fluorescence photography has not been found. The ideal filter for this would be one that absorbed all ultraviolet radiations and transmitted all the visible light. The Corning filter No. 038 Noviol shade A was almost ideal, but was itself highly fluorescent. However, this filter has been used successfully by placing it behind the lens inside the bellows where it is protected from most of the near-ultraviolet rays. The Corning filter No. 306 Noviol shade O, although almost ideal, transmits a negligible amount of near-ultraviolet and absorbs a trace of violet and blue. The Wratten No. 2A gelatin filter, which is used most frequently and is recommended by Walter Clark<sup>3</sup> for fluorescence photography, is also good. This does transmit some near-ultraviolet, but absorbs less violet and blue in the visible spectrum than the Corning No. 306. The Wratten No. 2 Aesculine gelatin filter also absorbs ultraviolet radiations shorter than 3,800 to 3,900 A.U. but this, like the Corning No. 038 Noviol A, is intensely fluorescent. When the primary filter is a glass with characteristics of the Corning No. 587 red-purple ultra, the Corning No. 306 is superior to all other secondary filters since it absorbs a part of the purple light transmitted by the primary filter.

The photograph illustrated was taken with a 5 by 7 inch extension bellows camera equipped with a lens having a focal length of  $6\frac{1}{2}$  inches. Kodachrome film,  $3\frac{1}{4}$  by  $4\frac{1}{4}$  inches, was used in a reducing back. Similar pictures have also been taken with several other cameras, including a Leica. In general, it may be said that almost any ordinary camera may be used for fluorescence photography. A quartz lens is not required and, as a matter of fact, would be a disadvantage because this would readily transmit the ultraviolet radiations. The glass lens in an ordinary camera also transmits some of the longer wave lengths or near-ultraviolet, but less than the quartz lens would transmit. The camera lens filter is thus necessary to absorb the reflected near-ultraviolet radiations (so-called actinic rays) which are not absorbed by the glass lens.

There is little information available with regard to the fluorescence of camera lenses. It is known, however, that the fluorescence characteristics of glass change with age, and that optical glasses or lenses from different manufacturers show unmistakable variations in fluorescence.<sup>4</sup> The differences in the color of the fluorescent light emitted by various lenses range from pale yellow to violet, blue, and brilliant white. The differences in shade have been described as subtle but so characteristic that glasses from numerous sources almost never fluoresce similarly, and this fact has been used by one manufacturer to distinguish between his own and other products.<sup>4</sup>

The discussion of the fluorescence of glass has been introduced to emphasize the importance of checking this feature in every camera lens before attempting to use it for fluorescence photography. During the long exposure necessary for





Fig. 1.



Fig.

A.

PLATE I

B.

FIG. 1 A and B.—Illumination. Visible light. A. Normal rat with the hardyrian gland exposed by removal and displacement of the eyeball. B. Rat maintained on a diet deficient in pantothenic acid. Note the incrustation of material that resembles dried blood. Illumination, incandescent tungsten filament (500 watt) at 5 feet. Camera, 5 by 7 inch extension bellows with a lens having a focal distance of  $6\frac{1}{2}$  inches. Kodachrome type B film. Exposure time,  $2\frac{1}{2}$  minutes at  $f:15$ .

FIG. 2 A and B.—Same as Fig. 1 A and B, but with near-ultraviolet illumination. Note the red fluorescence of the hardyrian gland and the porphyrin incrustations. Illumination, two 100 watt G. E. BH4 mercury arc lamps with an envelope of Corning No. 557 filter glass at 20 inches from the rats. Camera, same as above, 18 inches from rats to camera lens. Kodachrome daylight type film. Secondary filter, Wratten No. 2A for entire exposure. Corning No. 17 blue-green filter for last 4 minutes of exposure. Exposure time, 12 minutes at  $f:8$ .



Kodachrome photography a camera lens that fluoresced appreciably would cause fogging of the film or plate. This point was checked before making the color photograph in Plate I. The lens was not fluorescent so the filter to absorb the near-ultraviolet was placed between the lens and the plate. Had the lens been found to fluoresce, the filter would have been mounted in front of the lens. This would, therefore, have served a double purpose. It would have prevented ultraviolet radiation from reaching either the lens of the camera or the photographic plate. To test a lens for fluorescence irradiate it with near-ultraviolet light. If it is distinctly fluorescent, the lens will be seen to glow. Slight fluorescence may be detected by intermittently shielding the lens from the ultraviolet radiations by an ultraviolet-absorbing filter. If there is a glow which disappears when the lens is shielded, the lens is fluorescent. Shielding the lens with a hood or filter decreases the intensity of the fluorescence and the possibility of objectional effects.

There is great variation in the intensity as well as the wave length of the fluorescent light emitted by different materials. These widely varying conditions make it impossible to recommend specific types of Kodachrome film for recording fluorescence. In general, it may be stated that Kodachrome professional daylight type film is superior for objects which fluoresce a blue white, or blue and green with a little red. For objects that fluoresce more intensely in the red or yellow end of the visible spectrum, the type B professional Kodachrome gives the best color balance. Likewise, no specific rule can be given for the exposure time, except that it must always be relatively long in comparison to exposures with reflected light, because the intensity of the visible light resulting from the fluorescence is low and varies in different objects. Several factors will influence this variable fluorescence intensity in a definite way. The intensity of the light and its distance from the object regulate the intensity of fluorescence. The distance of the object from the camera or film must also be taken into account in estimating the exposure time.

As a preliminary procedure to determine the proper exposure, a series of test exposures of five, ten, twenty, and forty minutes at f:8 may be made and from these results the proper exposure is approximately estimated. It is imperative in Kodachrome photography to have the proper exposure, since this determines the accuracy of the method of recording the colors.

The fluorescence of the background is also an important feature. The background should be either nonfluorescent or should fluoresce a color that will set off or emphasize the color of the fluorescent object. A dull blue or black (nonfluorescent) background is best for red fluorescent objects. A white or red fluorescent background almost completely masks the fluorescence of red fluorescent objects.

The wide variation in the intensity of fluorescent light from different materials makes it impractical to try to photograph more than one specimen at a time. If this becomes necessary, groups of specimens should be chosen that exhibit a uniform fluorescence with regard to brightness. If this is impossible, it would be better to try to photograph the objects individually and to combine the photographs. In the case of the photograph of the fluorescence of rat fur

and the harderian gland, both objects fluoresce brightly. The red fluorescence of the harderian gland is relatively more intense than the blue-green fluorescence of the fur. It was found that when the proper exposure (twelve minutes) was given for the fur, the intense red of the gland and porphyrin incrustations was overexposed. To avoid this the exposure was made in the usual way for eight minutes. At the end of that time a Corning No. 430 dark shade blue-green filter was introduced between the object and the lens for an additional four minutes. This filter transmits the blue-green light from the fur but practically none of the red from the gland and porphyrin. In other words, the blue-green light from the fur acted on the film for twelve minutes, while the red of the gland and porphyrin incrustations acted only eight minutes. This yielded a more nearly perfect exposure for both materials. Accordingly, it may be seen that it is possible to compensate for differences in the brightness of the fluorescence of two objects by means of colored filters applied during a certain fraction of the exposure. It should be emphasized, however, that this is possible only for two or three different materials or colors, and in cases where the fluorescence spectra do not overlap too much. A number of attempts to compensate for the variations in brightness by means of illuminating the objects with filtered visible light of low intensity proved unsuccessful.

#### SUMMARY

A Kodachrome photographic method is outlined for recording the more delicate shades of colors caused by the fluorescence of objects when subjected to near-ultraviolet radiation. Some form of quartz mercury arc is recommended as the light source. This light must have a primary filter (Corning glass filter No. 586 or No. 587) transmitting the near-ultraviolet light, and absorbing all or almost all of the visible light. Only two of the secondary or lens filters mentioned in this discussion are suitable and practical. They are the Corning No. 306 Noviol O and the Wratten No. 2A. These may be mounted either in front of or in back of the camera lens. When it is necessary to photograph objects exhibiting the fluorescence of different colors and intensities, a more nearly perfect exposure is attained by means of a colored filter which absorbs the color emitted by the object which fluoresces most intensely. Such a filter would transmit the colors emitted by the materials which fluoresce less intensely. This filter is applied only during the last third of the exposure. Some of the requirements of the camera and lenses are also discussed.

Through the use of these methods and principles, it is possible to record faithfully on Kodachrome film some of the striking color effects brought to light by fluorescence studies.

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# DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**RESPIRATORY INFECTIONS, A Medium for Blood Cultures in, Humbert, C. R. Am. Rev. Tuberc. 45: 456, 1942.**

The following formula has been found very serviceable:

|                 |              |            |
|-----------------|--------------|------------|
|                 | Bouillon     |            |
| Peptone         |              | 10 Gm.     |
| Sodium chloride |              | 5 Gm.      |
| Lung infusion   |              | 1,000 c.c. |
|                 | Solid Medium |            |
| Agar-agar       |              | 15 Gm.     |
| Sodium chloride |              | 5 Gm.      |
| Lung infusion   |              | 1,000 c.c. |

If the powdered lung is used, 120 Gm. in 1,000 c.c. of water are soaked overnight in the ice chest. It is then boiled and filtered. This filtrate is used as described above. The reaction is set at 7.5 pH.

This medium has been useful in obtaining blood cultures in respiratory infections where others have failed. It is also used for many other organisms by adjusting the reaction suitable for their growth.

**TUBERCULOSIS, Vitamin K in, With Special Reference to Pulmonary Hemorrhage, Levy, S. Am. Rev. Tuberc. 45: 377, 1942.**

Vitamin K and its relation to pulmonary tuberculosis and pulmonary hemorrhage are discussed.

Bleeding time, coagulation time, clotting power, and liver function tests were carried out in four groups of patients with pulmonary tuberculosis and in one control group.

Of the patients with pulmonary tuberculosis 32 per cent had a more or less marked degree of hypoprothrombinemia, while there was no evidence of this condition among the subjects of the control group. Hypoprothrombinemia is a frequent finding in pulmonary tuberculosis and reaches the lowest values among the group of patients with pulmonary hemorrhage.

Parenchymal liver damage was found to be present in 57 per cent of patients with pulmonary tuberculosis, while 33 per cent of the control subjects showed a slight degree of liver dysfunction.

The effect upon the clotting power of drugs routinely used in pulmonary hemorrhage was studied. It was found that sodium pentobarbital causes a lowering of the clotting power. Morphine, koagamen, and calcium also have a slight beneficial effect on the clotting power, although inferior to that of vitamin K.

It is suggested that vitamin K be used routinely as a prophylactic measure in patients whose x-ray films show cavity formation and who are shown to have a hypoprothrombinemia.

Vitamin K, together with the administration of morphine, appears to be the therapy of choice in the prevention and treatment of pulmonary hemorrhage due to tuberculosis.

**BLOOD VELOCITY, An Objective Method of Determining (Fluorescein Method), Fishback, D. B., Gullman, S. A., and Abramson, E. B. Am. J. M. Sc. 203: 535, 1942.**

A new objective circulation time is described, using fluorescein.

The data obtained indicate that the method is reasonably simple, not harmful to the patient, and reliable as indicated by comparison with other methods.

This method is of particular advantage in determining the blood velocity in small children, and in comatose, anesthetized, mentally ill, and moribund patients.

In patients with normal hearts or with fully compensated cardiac disease, the circulation time varied from 7 to 15.6 seconds. In another group of patients with cardiac disease undergoing decompensation, the circulation time varied from 16 to 25 seconds, one being 45 seconds.

The test for circulation time is done as follows: Using sterile precautions, an 18 gauge needle attached to a syringe containing 4 c.c. of a 10 per cent, or preferably 3 c.c. of a 20 per cent, sodium fluorescein solution is inserted into the antecubital vein of the arm. The room is then darkened, and the fluorescein is injected rapidly into the vein. The assistant, using a portable ultraviolet source with a Wood's filter, directs the light to one of the eyes of the patient, observing the palpebral conjunctiva. The first appearance of a brilliant yellow color in the lower palpebral conjunctiva is the end point. The end point is sharp, especially if the room is dark. The time elapsed from the moment of injection to the appearance of the dye in the eye is recorded.

Thus, the circulation time from the antecubital vein of the arm to the conjunctiva of the eye is accurately determined.

#### **SYPHILIS, Value of Complement Fixation and Agglutination With Spirochetal Antigens and Relation of Spirochetal Antibody to the Wassermann Reaction, Kolmer, J. A. Arch. Dermat. & Syph. 45: 455, 1942.**

Complement fixation tests conducted with antigens of cultivated *S. pallida*, especially of the Reiter and Kazan strains, compare favorably in sensitivity with the Wassermann and flocculation tests in the serologic diagnosis of syphilis of human beings and rabbits, but their practical value in the diagnosis of the disease in its different stages and in relation to treatment has not been sufficiently defined.

Spirochetal complement fixation tests in syphilis of human beings and rabbits are not as specific as the Wassermann and flocculation tests, largely because of the presence in normal serum of natural spirochetal antibody.

This natural antibody, as well as that produced in syphilis, is of a group character reacting not only with cultivated *S. pallida* but with *S. macrodentium*, *S. microdentium*, and other spirochetes.

Spirochetal complement fixing antibody is increased in malaria and in leprosy, with a high incidence of positive complement fixation reactions in both diseases, especially in the former. Under the conditions it is improbable that the spirochetal complement fixation test will identify as biologic false reactions the positive Wassermann and flocculation reactions observed in nonsyphilitic persons with leprosy and malaria.

Normal human and rabbit sera also contain a natural group agglutinin for cultivated *S. pallida* and other spirochetes. The agglutinin undergoes some increase in syphilis but not sufficient for rendering the spirochetal agglutination test with cultivated spirochetes of diagnostic value in syphilis.

The results of agglutination and complement fixation tests with suspensions of virulent *S. pallida* obtained from acute testicular syphilomas of rabbits have indicated more definitely the production of these antibodies in syphilis of human beings and rabbits than tests employing suspensions of the cultivated spirochetes, which suggests that the latter have undergone dissociation into variants with a change in antigenic structure associated with a loss of virulence.

Complement fixation and agglutination tests with cultivated spirochetes have indicated the possible existence of serologic strains of *S. pallida* and also their biologic relation to *S. macrodentium*, *S. microdentium*, and *S. refringens*.

Largely on the basis of absorption of syphilitic human serum with tissue lipids and suspensions of cultivated *S. pallida*, the preponderance of evidence is in favor of regarding the Wassermann reagent and spirochetal antibody as separate entities which may coexist in serum.

Undoubtedly the lipids of *S. pallida* are important in complement fixation by the Wassermann reagent. Whether spirochetal antibody reacts with them is unknown, but it

has been suggested that it fixes complement with some other constituent of spirochetes of a nonprotein nature.

The practical value of antigens of cultivated *S. pallida* in the serologic diagnosis of syphilis and in relation to the treatment of the disease cannot be stated at present, but they are worthy of further study. If such strains are employed, antigens of whole spirochetes in phenolized saline solution appear to be advisable. The Reiter or Kazan strains are preferred, and a mixture of the two may be advisable. The amount employed should be the minimum, giving positive reactions with human syphilitic serum, in order to avoid falsely positive reactions with natural spirochetal antibody as much as possible. Suspensions of virulent tissue spirochetes are preferred, but owing to technical difficulties in their preparation they probably cannot be employed.

**CULTURE, Differential, Simple Method of Using Penicillin, Tellurite and Gentian Violet for, Fleming, A.** Brit. M. J., May 2, p. 547, 1942.

By the simple method of spreading a small quantity of gentian violet over half of the culture plate of blood agar after it has been planted, it is possible to isolate a few streptococci from the midst of a multitude of staphylococci, just as it has been shown to be if the gentian violet is incorporated in the medium.

Whatever chemical is used with this technique, it is necessary, when spreading it over half the plate, to start where the inoculum is lightest and proceed toward where it is heaviest; otherwise there may be so many resistant bacteria that a confluent sheet of growth results.

Three chemicals can be used in this way with great advantage in the clinical bacteriological laboratory. They are penicillin, potassium tellurite, and gentian violet.

The great advantages of this simple method of spreading the chemical over half the culture plate after inoculation are (1) special media need not be prepared; (2) one-half of the culture plate is an ordinary culture and the other half a selective culture, thus really giving the effect of two culture plates in one.

**GLUCOSE, The Assimilation Rate of Intravenously Injected, in Hospital Patients, Cain, J. C., and Belk, W. P.** Am. J. M. Sc. 203: 359, 1942.

The assimilation rate of intravenously administered glucose in hospital patients was found to be correlated with the rate of injection. The assimilation of 5.77 Gm. per kilogram of body weight per hour was observed.

In all cases some glucose was lost in the urine, the amounts varying from approximately 0.3 to 30 per cent of the total given. The average loss was 12.2 per cent.

The proportion of injected glucose lost in the urine was not correlated with the rate of injection, nor with the total amount given within the limits studied. This "spill" is conditioned by factors within the individual patients.

For practical purposes the rate for the intravenous administration of glucose may be determined solely on two considerations; first, the hydrodynamics of the patient's circulation; second, technical convenience.

**SYRINGE, Removing "Frozen" Plungers by a Hydraulic Pressure Method, Novak, M., and Laey, A. M.** Am. J. Technol. 8: 42, 1942.

A 1 inch No. 22 Yale hypodermic needle is telescoped into a No. 19 (1½ inch) Yale needle so as to make a firm connection. One needle is attached to the frozen syringe, and the other to the tuberculin syringe which is filled with water. By forcing water into the "frozen" syringe, pressure is exerted on the "frozen" plunger and almost always it will be forced out. Air in the system should first be displaced with water for best results.

This simple device may be made in a minute from old needles, and kept on hand for use when needed. Any combination of needles may be used as long as they fit together firmly. The use of the two needles in this manner makes a flexible connection and diminishes the chances of breaking the ends of the syringes.

**TISSUE, Combined Frozen and Paraffin Method for Rapid Sections, Thomlinson, B. C.**  
Arch. Path. 33: 35, 1942.

### *Technique*

1. The tissue, if not already fixed, is placed in hot dilute formaldehyde solution (10 c.c. of solution of formaldehyde U.S.P. in 90 c.c. of water) for three minutes. The solution should be steaming (about 65° C.) but not boiling, and the pieces of tissue may be large, but not over 3 mm. in thickness.

2. Frozen sections, as thin as possible (10 to 15 microns), are prepared by the usual method. Overfreezing should be avoided since it causes an unnecessary delay.

3. Sections are transferred from the knife to a dish of warm (40° C.) 0.2 per cent gelatin solution, from which a section is then floated carefully onto a clean slide, and any wrinkles which may form are teased out.

4. The excess fluid around the section is wiped off, and the slide is dried cautiously over the pilot flame of a Bunsen burner. Overheating is to be avoided.

5. When the section is thoroughly dried, it is cooled and placed in formaldehyde alcohol (10 c.c. of solution of formaldehyde U.S.P. in 90 c.c. of 95 per cent ethyl alcohol) for one-half minute.

6. It is then dehydrated in 95 per cent ethyl alcohol, followed by absolute alcohol, one-half minute in each.

7. After being cleared in xylene for one-half minute, it is transferred to a beaker of melted paraffin in an oven at 60° C. for three minutes.

8. The slide is then removed from the beaker of paraffin and returned to the oven for five minutes. This assures attachment of the section to the slide during the staining process and apparently produces a mordant effect on the tissues.

Various staining methods may now be employed. The technique for the hematoxylin-eosin method is as follows:

1. Remove paraffin with xylene for 30 seconds.

2. Place in 95 per cent alcohol for 15 seconds.

3. Place in 70 per cent alcohol for 15 seconds.

4. Wash quickly in tap water for 10 seconds.

5. Stain in fresh Harris hematoxylin for one minute.

6. Wash in water, and decolorize in 0.5 per cent aqueous solution of hydrochloric acid. Wash and intensify in a weak solution of ammonia water and again wash for 30 seconds.

7. Counterstain with eosin and rinse in water for one minute.

8. Dehydrate in 70 per cent, 95 per cent, and absolute alcohol for 10 seconds each.

9. Clear in xylene.

10. Mount in Canada balsam.

**HEMOSIDERIN, A New Modification of Perls's Reaction for, in Tissues, Highman, B.**  
Arch. Path. 33: 937, 1942.

It is suggested that one volume of a 2 per cent aqueous solution of potassium ferrocyanide be mixed with an equal volume of a 4 to 6 per cent aqueous acetic acid, heated in a test tube until beads of gas appear on the inner surface of the glass (about 60° to 80° C.), poured on the section, and allowed to remain thereon for from forty-five seconds to five minutes.

After being washed in water, the section is counterstained for five minutes with a 1:6,000 to 1:10,000 solution of basic fuchsin in 2 per cent aqueous acetic acid.



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